MONITORING OF THE PREVALENCE OF AVIAN INFLUENZA AND CHARACTERIZATION OF THE VIRUS ISOLATES IN KHARTOUM STATE

BY

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Dedication

To the late Professor Mohamed Elnasri Hamza who inspired my work and dedicated his life for advancement of science

To my parents, Mohammed and Salwa

Who always taught me to excel

My brothers and sisters

With love

PREFACE

This work was carried out in the Department of Preventive Medicine and Public Health, Faculty of Veterinary Medicine, University of Khartoum, Central Veterinary Research Laboratories and the Institute of Endemic Diseases under the supervision of Professor Mohamed ElNasri Hamza and co-supervision of Professor Muawia Mohamed Mukhtar and Professor Mahasin El Nour Abd Elrahman.

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List of abbreviations

HPAI	Highly Pathogenic Avian Influenza
LPAI	Low Pathogenic Avian Influenza
AI	Avian Influenza
AIV	Avian Influenza Virus
ND	Newcastle Disease
HA	Hemagglutination
HI	Hemagglutination Inhibition
NA	Neuraminidase
CEF	Chick Embryo Fibroblast
CKC	Chick Kidney Cell
MDCK	Madin – Darby Canine Kidney Cell
ELISA	Enzyme Linked Immunosorbant Assay
AGID	Agar Gel Immunodiffussion Test
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RRT-PCR	Real Time Reverse Transcriptase-Polymerase Chain Reaction
NASBA	Nucleic Acid Sequence Base Amplification
MP	Matrix Protein
NS	Non-Structural protein
PB1	Polymerase Base 1
PA	polymerase
MLD_{50}	Minimum Lethal Dose 50
mRNA	messenger RNA
cRNA	complementary RNA
T.S	Tracheal Swabs
C.S	Cloacal Swabs

T	Tissues
PBS	Phosphate Buffer Saline
PD	Phosphate Diluent
RBCs	Red Blood Cells
GMEM	Growth Minimum Essential Medium
TPB	Tryptose Phosphate Broth
PCV	Packed Cell Volume
AF	Allantoic Fluid
OIE	Office International des Epizooties

ABSTRACT

This work was designed to investigate the prevalence of avian influenza (AI) in Khartoum State during the period 2004 – 2007. Attempts were made for antibody detection and virus isolation, identification, characterization and determination of the pathogenicity of the isolates. Isolated viruses were subjected to biological and physiochemical characterization and plaque production in the cell culture.

A total of 516 blood samples were collected from randomly selected chickens from different localities in Khartoum, Khartoum North and Omdurman. Two hundred and fifty four blood samples were collected during the period 2004-2005, while 262 blood samples were collected in 2007 after occurrence of the suspected outbreak of the disease. These sera were examined for the presence of antibodies against AI using Agar Gel immunodiffusion (AGID), Enzyme-Linked Immunosorbant Assay (ELISA) and Hemagglutination Inhibition Tests (HI). In addition, isolation and identification of the causative Avian Influenza Virus (AIV) was done from 50 tissues, 100 cloacal and 100 tracheal swabs collected before and after the outbreak. Virus identification was done by AGID and HI. In these samples, AIV was also confirmed by nucleic acid amplification technique, RT-PCR.

The results showed that 37% and 16% of the tested sera were positive by ELISA for type A before and after the outbreak, respectively, 9% and 4.2% were positive by AGID before and after the outbreak for H5N2, respectively. The positive sera for AIV were further subtyped by the HI test using H5N3 and H7N1 antigens. The results of sera collected in 2004-2005 revealed that 60.7% were positive for H5N3 and 39.3% for H7N1, while 100% were positive for H5N3 from sera collected in 2007.

A total of 5 virus isolates were isolated from samples in the allantoic fluid of 9-11-day old embryonated eggs. These isolates agglutinated chicken and horse RBCs and produced plaques in tissue culture, indicating that the virus is the highly pathogenic type.

The biological and physiochemical properties of isolates were recorded for the first time for avian influenza virus in the Sudan. Generally the results indicated that H5 AIV is circulate in the field of study; however N1 was not detected due to the lake of specific primers required for detected of that gene.

المستخلص

صممت هذه الدراسة لمعرفة مدى انتشار انفلونزا الطيور بولاية الخرطوم فى الفترة 2004-2007 من خلال الكشف عن الاجسام المضادة و عزل الفيروس و التعرف عليه و تحديد امراضية العترات المعزولة تجريبيا و ايضا دراسة التغيرات البيولوجية و الفيزوكيميائية على الفيروس من حيث نموه و انتاج اللطعات فى الزرع النسيجى.

تم جمع 516 عينة دم من دواجن اختيرت عشوائيا من مناطق مختلفة فى الخرطوم والخرطوم بحرى و امدرمان. جمعت 254 عينة دم فى الفترة 2004-2005 و جمعت 262 عينة فى العام 2007 و ذلك عقب ظهور وباء انفلونزا الطيور فى السودان. فحصت عينات السيرم للكشف عن وجود الاجسام المضادة للمرض باستخدام اختبار الترسيب فى الاجارو اختبار الاليزا و اختبار تثبيط تلازن كريات الدم الحمراء .بالاضافة الى محاولة عزل الفيروس و التعرف عليه من 50 عينة نسيج ، 100 مسحة من المجمع و 100 مسحة من الرغامى قبل وبعد الوباء و امكن التعرف على الفيروس بواسطة اختبار الترسيب فى الاجار و اختبار تثبيط تلازن كريات الدم الحمراء. تم تأكيد وجود فيروس انفلونزا الطيور بواسطة تقنيات الحمض النووى منها تفاعل البلمرة المتسلسل الناسخ الرجعى.اظهرت النتائج ان 37% و 16% من عينات السيرم كانت موجبة لاختبار الاليزا للنوع A قبل و بعد الوباء على التوالى و 9% و 4.2% كانت موجبة لاختبار الترسيب فى الاجار قبل و بعد الوباء و ذلك لانتجين H5N2 و اختبرت عينات السيرم الموجبة لتحديد العترات بواسطة اختبار تثبيط تلازن كريات الدم باستخدام الانتجينات H5N3 و H7N1.

اظهرت نتائج عينات السيرم التى جمعت فى2004-2005 ان 60.7 % موجبة للانتجين H5N3 و 39.3 % للانتجين1 H7N و 100% موجبة للانتجينH5N3 من عينات السيرم التى جمعت فى العام 2007. عزلت 5 عترات للفيروس من العينات فى السائل السقلئى لاجنة االبيض عمر 9-11 يوم،احدثت العترات تلازن لكريات الدم الحمراء للدواجن و الحصان و انتجت لطعات فى الزرع النسيجى مما يدل على أن الفيروس من النوع شديد الضراوة.

درست الخواص البيولوجية و الفيزيوكيميائية للعترات المعزولة لاول مرة لفيروس انفلونزا الطيور فى السودان . اوضحت النتائج أن فيروس انفلونزا الطيور التى تم عزلها تحتوى على الجين H5و أن الجين N1 لم يحدد لعدم توفر البادئات الخاصة لهذا الجين.

INTRODUCTION

Poultry production is very important in Sudan as a source of dietary protein and as a source of income. The marketing of chickens in live bird markets is a common practice in many regions of the State and this presents particular biosecurity challenges and risks to human health.

Respiratory diseases play an important role in poultry and result in substantial economic losses to the poultry industry (Glisson, 1998 and Villegas, 1998).

Avian influenza (AI) is a serious viral disease of domestic poultry and other avian species included in OIE list A (OIE, 2006). The disease affects most types of birds and occurs as epidemics in poultry farms. The fatal disease is named “Highly Pathogenic Avian Influenza (HPAI) “.

Highly pathogenic avian influenza strains are capable of causing severe economic losses including drop of egg production , mortality (as high as 100%) of infected chicken flocks. It caused large epidemics that devastated the poultry industry in many countries over the last few years. and posses serious risk to human health.

The disease has worldwide distribution which varies in severity from mild to an acute fatal disease of chickens, turkeys and other avian species.

Clinically the disease is similar to infectious bronchitis, infectious laryngotrachitis, fowl cholera and all forms of Newcastle disease (Jacob *et al* , 1998).Since 1995 AI has became a very important disease problem throughout the world.Until recently, AI was not a disease commonly found in chicken flocks, but lately it became necessary for a large scale mobilization of federal and State regulatory efforts aimed at eradication.

Recently, however, avian influenza acquired world-wide attention when a highly pathogenic strain of the subtype H5N1, which probably arose before 1997 in Southern China, gained enzootic status in poultry throughout South East Asia and unexpectedly crossed host barriers (Perkins and Swayne, 2003) infecting mammals (cats, swine, humans).

The recent outbreaks of the disease worldwide highlighted the difficulties in controlling this disease both in developed and in developing countries. Biosecurity is considered the most important tool to prevent and control AI.This disease may have a devastating effect on the poultry industry particularly following the high mortality rates in susceptible birds, but also its presence in a given territory results in restrictions on animal movements, marketing and trade of poultry products (Ilaria *et al*, 2002).

The disease was reported in the Sudan for the first time in 1923 (Report of the Sudan Veterinary Service, 1923) since then, attempts were made to study AI in the Sudan. El Amin (2000) demonstrated antibodies to influenza virus in the sera of chickens and isolated influenza virus type A; this was the first record of isolation of influenza virus in the Sudan. Prior to that EL Amin and Kheir (1985) reported the presence of antibodies to influenza virus type A in the sera of camels, goats, sheep and cattle in Kassala area. The HPAI type A H5N1 was first reported in the Sudan in April 2006.

Due to the expanding poultry production in the Sudan, the public health importance of the disease and scarcity of information about this serious disease that causes great economic losses, it was considered necessary to carry out the proposed research work.

This present work was carried out to:

1. Determine the presence of antibodies to influenza virus before and after an outbreak that occurred in Khartoum State during the period 2004 to 2007.
2. Study the clinical and pathological changes, biological and physiochemical characters among naturally infected chickens.
3. Isolate, identify, characterize and determine the pathogenicity of the isolates.

CHAPTER ONE

REVIEW OF THE LITERATURE

1.1 Avian influenza

Avian influenza (AI) is a contagious viral infection of many avian species worldwide including domestic poultry, wild and exotic birds, shore birds and migratory waterfowl. The disease prevalent is caused by the highly pathogenic viruses of the H5N1 subtype and is present in many countries in Asia, Europe, Middle East and Africa (Maria *et al* , 2007), The clinical forms vary from a very mild to a highly fatal disease. In mild infection, no symptoms of illness may be recognized. The disease has an incubation period of 24 hours to 7 days depending on the dose of the virus and route of entry (David et al , 2003; Easterday and Beard, 1984).

There are numerous strains or subtypes of avian influenza viruses. Most of these viruses are associated with subclinical or mild to moderate respiratory infections characterized by coughing and sneezing. Sinusitis may develop and infected birds often experience decreased egg production. The highly pathogenic forms of avian influenza (fowl plague) is presented as severe generalized disease with high mortality in commercial flocks. Deaths may occur as early as 24 - 48 hours after the onset of clinical signs. Comb and wattles are cyanotic and there may be an edema of the head region with

coughing, gasping, blood-stained oral and nasal discharges, and diarrhea (Carter *et al* , 2006). Lesions include hemorrhages and congestion of serous and mucous membranes, consolidation of lungs, and caseation involving the air sacs. Focal necrosis may be noted in the skin and internal organs.

Avian influenza viruses of 15 subtypes can cause low pathogenicity avian influenza (LPAI) in susceptible birds. This is, a mild respiratory disease with low mortality rates in poultry. However, in some cases, the infection may cause significant mortality rates, during coinfections with other bacterial or viral infections. The severity of the clinical condition caused by LPAI viruses does not correlate with the viral subtype, as clinical conditions associated with decreased performances and symptoms affecting the respiratory, reproductive or enteric tracts have been observed with H1 (Ficken *et al* ., 1989), H3 (Tang *et al* ., 2005), H5 (Shortridge *et al* ., 1998), H6 (Webby *et al* ., 2002), H7 (Capua *et al* ., 2000) and H9 (Nili and Asasi, 2003) viruses.

In chickens and turkeys, morbidity and mortality rates are variable as the signs and depend on virus pathogenicity, host, age and environmental conditions (Easterday *et al.*, 1997).

Infection of poultry with avian influenza viruses cause a wide range of clinical signs including asymptomatic infections, mild to severe respiratory disease, production losses, and rarely, severe with high morbidity and mortality.

1.2 History of the disease

Avian influenza was defined as “fowl plague” in 1878 as an infectious disease of birds causing high mortality in chickens in Italy (Perroncito 1878). Due to a former hot spot in the Italian Upper Po Valley it was also referred to as 'Lombardian disease'. Although Centanni and Savonuzzi, in 1901, identified a filtrable agent as responsible for causing the disease, it was not before 1955 when Schafer characterized these agents as influenza a viruses (Schafer, 1955). In the natural reservoir hosts of avian influenza viruses, wild water birds, the infection generally runs an entirely asymptomatic course where a virus biotypes of low pathogenicity co-exists (Webster et al., 1992, Alexander 2000).

The terminology “highly pathogenic avian influenza” was officially adopted in 1981 at the First International Symposium on Avian Influenza. The Office International des Epizooties (OIE) has included HPAI as a List A reportable disease (OIE, 2003).

Since 1997, outbreaks of highly pathogenic (HP) H5N1 and circulation of H9N2 viruses among domestic poultry in Asia have posed a threat to public health (Nguyen *et al*., 2005). In 2003-2004 outbreaks of HPAI caused by H5N1 viruses were reported in eight Asian countries (Sims *et al.*, 2005).

1.3 Host range

Wild aquatic birds, notably members of the orders *Anseriformes* (ducks and geese) and *Charadriiformes* (gulls and shorebirds), are carriers of several strains of influenza virus A subtypes, and thus, most probably constitute the natural reservoir of all influenza A viruses (Webster 1992, Fouchier et al., 2003; Krauss et al., 2004, Widjaja et al. 2004; Hinshaw et al., 1980; Suss et al ., 1994). While all bird species are thought to be susceptible to AI, some domestic poultry species - chickens, turkey, guinea fowl, quail and pheasants - are known to be highly vulnerable to the sequelae of infection. Avian influenza type A viruses generally do not cause disease in their natural hosts. (Taubenberger *et al.*, 2005).

Chickens and turkeys are highly susceptible to infection and clinical disease. Ducks and geese are susceptible to infection with all AI virus strains, but only some very virulent viruses produce clinical disease. Their potential as reservoir hosts is considered to make waterfowl a major source of virus for poultry. In 2004, subtype H5N1 caused widespread deaths in ducks, geese and chickens in China (WHO 2004). Previously, deaths were recorded in waterbirds in Hong Kong in 2002. There were reports of limited mortalities in ducks and geese in Italy (Capua and Mutinelli 2001; Sturm-Ramirez *et al.*, 2004).

1.4 Transmission and spread of the disease

The disease occurs in chickens, ducks, turkeys, quail, pheasants, other fowl and particularly in waterfowl, AI is highly contagious and spreads rapidly (Carter et al, 2006).

The virus replicates predominantly in the intestinal tract (Slemons and Easterday, 1975; Fouchier *et al* ., 2007), and is shed in faeces (Webster *et al* ., 1978; Hinshaw *et al* ., 1980) and is subsequently transmitted and maintained by faecal-oral transmission. Poultry trade and mechanical movement of infected materials are likely modes for spreading avian influenza (Alexander, 2000).

Migratory birds may spread AI (H5N1) viruses to new geographic regions, but their importance as an ecologic reservoir is uncertain. The spread of influenza A (H5N1) viruses appears to be principally related to the movement of poultry and poultry products, (Gauthier *et al* ,2007; Kilpatrick *et al* ,2006) although recent outbreaks of virus infection in sub-Saharan Africa,(Ducatez *et al* ,2006) Egypt, and Europe may indicate introduction of the virus by wild birds. The risk of the introduction of influenza type A (H5N1) viruses into North America by birds migrating through Alaska appeared to be low (Winker *et al* , 2007).

The risk that infection will be transmitted from wild birds to domestic poultry is greatest where domestic birds roam freely, share a water supply with

wild birds, or use a water or food supply that might become contaminated by droppings from infected wild bird carriers (Capua et al., 2003, Henzler et al., 2003).

The infection cycle among birds depends on faecal-oral transmission chains. Apart from being directly transmitted from host to host, indirect spread via virus-contaminated water and fomites is an important route in contrast to influenza virus infections in mammals (humans, swine, and horses) where transmission by aerosols prevails.

Birds are infected by direct contact with virus-excreting animals and their excretions or through contact with (abiotic) vectors which are contaminated with virus-containing material. Once introduced into domestic flocks, LPAIV may be secreted to sustained horizontal transmission within and between flocks. HPAIV spreads by similar means. The wet markets, where live birds are sold under crowded conditions, are multiplicators of spread (Shortridge et al., 1998, Bulaga et al., 2003).

1.5 Geographical distribution of the disease

Highly pathogenic H5N1 influenza viruses have repeatedly caused serious outbreaks of disease in poultry farms since 1997 and posed a significant threat to human health due to their ability to infect humans, resulting in high mortality (Claas et al.,1998; Subbarao et al.,1998, Peiris et al., 2004). Since late 2003, H5N1 viruses have spread in an unprecedented manner

across Asia, resulting in more than 60 human fatalities in Thailand, Vietnam, Cambodia, and Indonesia and in the slaughter or infectious deaths of more than 150 million birds. Despite extensive efforts to contain these outbreaks, H5N1 viruses continue to circulate among poultry in Asia (OIE, 2008) and remain a threat to both veterinary and human public health.

To date, all outbreaks of HPAI in domestic poultry have been caused by H5 or H7 influenza A subtypes. Until 1999, HPAI was considered relatively rare, with only 17 outbreaks reported worldwide between 1959 and 1998; however, since 1999 the number of outbreaks occurring globally has increased (Capua and Alexander ,2004, Capua et al., 2006).

Nigeria was the first African country to experience outbreaks of H5N1 in poultry (in February 2006). One study showed that three different sublineages were independently introduced into Nigeria through routes that coincide with flight paths of migratory birds, although the authors stated that independent trade imports could not be ruled out as the source of spread (Ducatez et al, 2006). Another study found that isolates from Nigeria were closely related to isolates from West Africa and Sudan (Fasina et al., 2008).

1.6 Clinical features

Infections of domestic avian species with low pathogenic avian influenza (LPAI) viruses can be asymptomatic or cause a wide range of clinical signs varying from mild respiratory disease to more severe diseases

affecting the respiratory and enteric systems. Highly pathogenic avian influenza viruses (HPAI) cause rapid mortality in poultry, which often approaches 100% of incidence (Alexander, 2000).

1.7 Lesions

According to Saif (2003) the lesions of the disease are variable in their location and severity, depending on the host species, pathogenicity of the infecting virus and presence of secondary pathogens. The lesions in HPAI include edema, hemorrhage, and necrotic foci in liver, spleen, kidneys, intestine and pancreas (Easterday and Beard, 1984: Jungheer, 1946).

1.8 Diagnosis of avian influenza

The recent emergence and re-emergence of influenza viruses with pandemic potential is of great concern to both the veterinary and public health communities. Early diagnosis of influenza virus infection is therefore essential.

Clinically the disease can be confused with other poultry diseases. Thus definite diagnosis depends on isolation and identification of the virus (Jordan, 1990).

1.8.1 Isolation and Identification

Isolation of influenza virus was performed by inoculating 9-day-old embryonated chicken eggs with 0.2 ml tissue suspensions or swab suspensions via the allantoic cavity. The eggs were incubated for 4 days and candled daily for viability; embryos that died within 24 hours of inoculation were discarded

as nonspecific. Allantoic fluids from dead and surviving embryos were tested for Hemagglutination (HA) activity. Samples that yielded no hemagglutination were re-inoculated for a second passage.

OIE (2008) described the standard methods for isolation of the virus in chicken embryo. Also suggested the following criteria for identification of virus:

- Detection of HA activity indicates a high probability of the presence of an influenza A virus. Fluids that give a negative reaction should be passaged into at least one further batch of eggs.

- Several serological and molecular methods are available to confirm the presence of influenza A virus; and include:

  - Agar gel immunodiffusion (AGID) tests that demonstrate the presence of the nucleocapsid or matrix antigens
  - Enzyme-linked immunosorbent assays (ELISAs)
  - Reverse-transcription polymerase chain reaction (RT-PCR) using nucleoprotein-specific or matrix-specific conserved primers; the presence of subtype H5 or H7 influenza virus can be confirmed by using H5- or H7-specific primers

1.8.1.1 Hemagglutination (HA) and Hemagglutination Inhibition test (HI).

1.8.1.1.1 Hemagglutination (HA)

Influenza viruses hemagglutinate avian and mammalian erythrocytes. The hemagglutinin (HA) protein agglutinates erythrocytes, hence the derivation of its name. The traditional method for identifying influenza field isolates takes advantage of this property (WHO, 2002).

1.8.1.1.2 Hemagglutination Inhibition test (HI).

The test is a subtype specific test that measures the ability of test serum to block the hemagglutination of constant amount of virus (Suarez and Cherry, 2000).

For the avian influenza (AI) virus, the HI assay is used to identify the hemagglutinin (H) subtype of an unknown AI virus isolate or the HA subtype specificity of antibodies to AI virus. Since the HI assay is quantitative, it is frequently applied to evaluate the antigenic relationships between different AI virus isolates of the same subtype. The basis of the HI test is inhibition of hemagglutination with subtype-specific antibodies (Janice and Rederesen, 2008).

The hemagglutination inhibition (HI) assay is also a widely used serological method to measure the levels of protective antibody responses against influenza viruses. However, the traditional HI assay which uses

chicken erythrocytes is not sufficiently sensitive for detecting HI antibodies specific to avian influenza viruses. Jia *et al* (2008) demonstrated that employing an assay using horse erythrocytes increased the sensitivity of detecting HI antibodies specific for three major serotypes of avian influenza viruses. It is more sensitive than complement fixation and more specific in differentiating between HA subtypes (Julkunen *et al* , 2002)

1.8.1.2 Agar Gel Immunodiffusion (AGID)

The agar gel precipitation test is group-specific test and is used to confirm positive sera (Peter, 2006).The test is primarily used to detect group specific antibody, and measure antibody to both NP and M1 proteins and was widely used for testing chickens (Beard, 1970) and it is more sensitive than ELISA and HI test (Snyder *et al* , 1985; Meulemans *et al* , 1987).

1.8.1.3 ELISA

Enzyme-linked immunosorbent assays (ELISA) are sensitive and specific ELISA that demonstrates nucleoprotein of type A influenza virus using a monoclonal antibody against type A influenza nucleoprotein (Slemons and Brugh, 1998; Swayne *et al*., 1998).

1.8.2 Molecular techniques

1.8.2.1 Reverse transcriptase PCR (RT-PCR)

Influenza diagnostic methods based on reverse transcription-PCR (RT-PCR) and real-time RTPCR (RRT-PCR) are currently available for HA, but they are not well developed for NA identification (Poddar, 2002; Takao et al., 2002)

Several different methods, including traditional reverse transcription-polymerase chain reaction (PCR), real-time reverse transcription-polymerase chain reaction, and nucleic acid sequence-based amplification among others, have been described for the diagnosis of avian influenza in poultry (David *et al* ., 2007).

Enveloped particles of influenza A viruses harbour eight segments of single-stranded genomic RNA of negative polarity. Two of the eight segments encode the envelope glycoproteins haemagglutinin (HA) and neuraminidase (NA), whose antigenic properties are used to distinguish influenza virus subtypes (Fouchier *et al* ., 2005).

Ming *et al* (2001) found that RT-PCR generated results that were highly consistent with the serological methods; moreover, RT-PCR could be used for

the identification and HA-subtyping of avian influenza viruses directly from organ homogenates.

RT-PCR has been used to differentiate H1 from H3 virus (Wright *et al* ,1995 and Stockton,1998), or to differentiate N1 from N2 virus (Stockton *et al* ., 1998). Moreover, RT-PCR followed by sequence analysis of the HA cleavage site was used for rapid determination of the virulence potential of H5 and H7 viruses in birds (Horimoto et al., 1995; Senne *et al.* , 1996). It is believed that PCR might serve as a fast and effective alternative to virus isolation for the detection of influenza A virus (Claas *et al* ., 1993; Yuen *et al* .,1998). However, to date there is no report on differentiating H1–H15 or N1–N9 of avian influenza viruses by RT-PCR.

Real-time RT-PCR (RRT-PCR) is a relatively new technology that has been used for avian influenza (AI) virus detection since the early 2000 for routine surveillance, during outbreaks, and for research. Some of the advantages of RRT-PCR are high sensitivity, high specificity, rapid and low cost. Furthermore, RT-PCR can be used with different sample types, is less expensive than virus isolation in chicken embryos, and since infectious virus is inactivated early during processing, biosafety and biosecurity are also easier to maintain (Eirca and David, 2008).

Comparative tests with throat swab samples from humans and fecal and cloacal swab samples from birds confirmed that the new PCR is faster and up to 100-fold more sensitive than classical virus isolation procedures (Ron *et al.,* 2000). Spackman *et al.,* (2002) found that the sensitivity and specificity of the H7- and H5-specific RRT-PCR were similar to those of virus isolation (VI) and hemagglutination inhibition (HI).

Hui-Ling *et al.,* (2006) suggested that the RT-PCR was rapid and specific and, therefore, could be valuable in the rapid detection of H5N1 influenza viruses in clinics. There are many different RT-PCR methods applied for AIV detection (Cherian *et al.,* 1994, Fouchier *et al.,* 2000, Lee *et al.*, 2001, Munch *et al.,* 2001, Starick *et al .,* 2000).

Real-time RT-PCR (RRT-PCR) for type A influenza detection is widely used with subsequent tests for subtype identification (Spackman and Suarez 2008).

1.9 Avian influenza virus

Avian influenza A viruses (AIV) are the causative agents of the currently most important poultry diseases.

The influenza A virus particle or virion is 80–120 nm in diameter and usually roughly spherical, although filamentous forms can occur (Jordan,

1990). Influenza A viruses have a segmented genome of single-stranded negative-sense RNA and belong to the family *Orthomyxoviridae* (Swayne *et al* ., 2000). The genome of influenza A virus consists of eight single-stranded, negative-sense genomic RNA (vRNA) segments that code for ten 10 distinctive proteins (Sigfrido and Sergio, 2007), associated with nucleoprotein (NP) and the viral polymerase complex (PB1, PB2 and PA) in the form of ribonucleoprotein complexes (RNP) (Portela, 2002). The proteins can be divided into surface and internal proteins. The surface proteins include haemagglutinin (HA), neuraminidase (NA) and two matrix proteins. The HA and NA proteins provide the most important antigenic sites for the production of a protective immune response, primarily in the form of neutralizing antibody. There is a great deal of antigenic variation among these proteins, with fifteen HA and nine NA subtypes being recognized, based on haemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests, respectively. (Swayne *et al*. , 2000).

The eight gene segments of influenza A virus encode 10 proteins: hemagglutinin (HA), neuraminidase (NA), matrix proteins M2 and M1, nonstructural (NS) proteins NS1 and NS2 and the three polymerases, the PB1 (polymerase basic 1), PB2, and PA (polymerase acidic) proteins (Webster *et al* ., 1992).

1.9.1 Classification

Avian influenza viruses are classified in the family *Orthomyxoviridae*, genus *Influenzavirus* (Lamb and Krug, 1996; David et al , 2003, Swayne et al , 2003: Ito *et al* ., 2001). The influenza viruses of this family include three genera: influenza A, B and C on the basis of the antigenic character of the internal nucleoprotein antigen. Only influenza A viruses have been isolated from avian species. Influenza A viruses are further divided into subtypes determined by haemagglutinin (H) and neuraminidase (N) antigens. At present, fifteen H subtypes and nine N subtypes have been identified.Each virus has one HA and one NA antigen, apparently in any combination. All influenza A subtypes in the majority of possible combinations have been isolated from avian species. To date, only viruses of H5 and H7 subtype have been shown to cause HPAI in susceptible species, but not all H5 and H7 viruses are virulent (Ilaria and Alexander, 2007).

Subtypes H5 and H7 have caused serious outbreaks of avian influenza in commercial flocks of chickens and turkeys (Carter *et al.*, 2006).

1.9.2 Pathogenicity

Influenza A viruses infecting poultry can be divided into two distinct groups on the basis of their ability to cause disease. The very virulent viruses cause highly pathogenic avian influenza (HPAI) that may result in mortality as high as 100%.These viruses have been restricted to subtypes H5 and H7,

although not all viruses of these subtypes cause HPAI. All other viruses cause a much milder disease consisting primarily of mild respiratory disease, depression and egg production problems in laying birds (Ilaria *et al.*, 2002).

There is a wide range of pathogenicity among the avian influenza viruses. Infections with viruses may be inapparent or result in disease that ranges from mild, transient syndrome to 100% morbidity or mortality. Signs of the disease may be respiratory, enteric or reproductive and will vary with virus, species, age, intercurrent infections, environment and immune status of the host (Esterday and Hinshaw, 1991)..

Depending on pathogenicity in chickens and turkeys, avian influenza A viruses are classified as virulent causing fowl plague or avirulent causing mild or asymptomatic disease.

There is extreme variation in virulence among subtypes of AI viruses, and a variety of subtypes are widespread throughout wild aquatic bird populations. HPAI due to H5 and H7 subtypes can cause severe clinical disease, and even subtypes of low pathogenicity, including H5 and H7, can be associated with severe clinical disease in the presence of other infectious agents. The pathogenicity of AI viruses depends on the genetic properties of the virus and the species of the host. Only viruses with H5 and H7 antigens have been isolated so far from HPAI in poultry. These two subtypes of AI virus are considered to be high risk strains for pathogenicity drift towards

HPAI, even if the clinical picture seen in poultry is of lesser or no pathogenicity. The cleavability of viral haemagglutinins by proteolytic enzymes also correlates with the virulence of virus strains for chickens.LPAI infections of chickens and turkeys with H5 and H7 subtype that have been allowed to continue without adequate control or eradication procedures have ultimately turned into virulent HPAI infections. The change in pathogenicity of the virus is associated with the acquisition of additional basic amino acids at the cleavage site of the haemagglutinin protein. In the controlled laboratory environment, HPAI was generated from an LPAI H5 subtype virus, derived from a water bird, after 24 passages through chickens (Ito *et al.*, 2001).

Most HPAI viruses isolated from poultry were from chickens and turkeys. Clinical signs result from the replication of the virus in the respiratory tract and subsequent systemic replication in the visceral organs and brain. The viruses that are nonpathogenic replicate only on the surfaces of the respiratory and intestinal tracts. The major determinant of pathogenicity of AI viruses is the cleavability of the H protein. If the H cleavage site has the right configuration of basic amino acids, the protease enzymes found in internal organs are able to cleave the protein. Without this configuration, however, the protein can only be cleaved by trypsin-like enzymes, which have a more restricted distribution on endodermal surfaces, such as the respiratory and intestinal tracts (Swayne and Suarez 2000).

Highly pathogenic avian influenza viruses are not necessarily virulent for all species of birds and the clinical severity seen in any host appears to vary with both bird species and virus strain (Alexander *et al.,* 1978; Alexander *et al.,* 1986) In particular, ducks rarely show clinical signs as a result of HPAI infections, although there are reports that some of the Asian H5N1 viruses have caused disease (Sturm *et al.,* 2005).

1.9.3 Properties of avian influenza virus

1.9.3.1 Physical properties

The influenza viruses are relatively unstable in the environment. Heat, extreme changes of pH, or nonisotonic conditions and dryness can readily inactivate the influenza viruses (Lamb, 1989)

Both AI and ND viruses can cause mild to severe disease in commercial poultry, including egg laying chickens, with virus being shed from the respiratory tracts and sometimes faeces. Both viruses can be partially protected from heat inactivation by the presence of organic material (Alexander, 2003; Swayne & Halvorson, 2003).

Orthomyxoviridae are considered to be sensitive to acid pH values, although their retention of infectivity is dependent on the degree of acidity that is obtained and the virus strain (Puri *et al .,* 1990).

Influenza virus may remain infective in lake water for up to 4 days at 22^{o}C and over 30 days at 0^{o}C (Webster *et al .,* 1978).

Data have demonstrated that an AIV H7N3 subtype at a concentration of 4 HA units in peptone water (pH 7.0) decreased its HA activity but preserved its infectivity when incubated at 4, 30 and 37°C for 35 days (*Muhammad et al* ., 2001). Infectivity of the same strain was also retained following exposure to higher temperatures, such as 56°C for 30 min but was lost after a prolonged exposure at 56°C for 60 min (Muhammad *et al* ., 2001). These data were ingreement with the studies on the resistance of four LPAI strains H7N2 subtype (Castro *et al* ., 1998; Lu *et al* ., 2003). The viruses, from 10^4 to 10^5 ELD_{50}/ml, retained their infectivity after 30 min at 56°C in a water bath and were completely inactivated after 60 min at the same temperature or after 10 min at 60°C (Castro *et al* ., 1998; Lu *et al* ., 2003).

King (1991) also evaluated the heat inactivation of AIVs at 56 and 60°C. Two LPAI strains (H5N9) and (H9N2) were selected to investigate the heat stability at 56 and 60°C. Both viruses were completely inactivated after 60 min at 56°C and after 30 min at 60°C.

Also inactivation of the virus occurred at temperature of 56^0C for 30 hr and 60^0C or more for 30minutes (OIE, 2002).

Lu *et al* (2003) studied the infictivity and inactivation of the H7N2 AIV in various environmental conditions, heat,pH and disinfectants, they found that

the virus was effectively inactivated at pH 2, heating at 56^0C and exposure to 70% ethanol in less than 30 min. Songserm *et al.,* (2006) studied the stability of H5N1 HPAI virus isolated in Thailand determining the survival of the infectious virus (initial dose of $10^{6.3}$ ELD_{50}/ml) mixed with chicken faeces under different environmental conditions. They concluded that virus was completely inactivated within 30 min after direct sunlight exposure.It can also be inactivated by heating to 56°C (133°F) for a minimum of 60 minutes (Center, 2007).

1.9.3.2 Chemical properties

The influenza viruses are relatively unstable in the environment. Heat, extreme changes of pH, or nonisotonic conditions and dryness can readily inactivate the influenza viruses.

1.9.3.3 Biological properties

The surface glycoproteins HA and NA are critical for the biology of influenza virus. HA is responsible for the virus attachment to the cell surface, binding to sialic acid residues in cell membrane glycoproteins, thus triggering viral fusion and entry (Takeda *et al.,* 2003). Some HA types can be cleaved by different proteases, what enables the virus to spread more efficiently *in vivo* (Gamblin *et al.,* 2004; Steinhauer, 1999). The antigenic diversity of HA and

NA provides for Influenza A virus subtyping. Fifteen HA and nine NA subtypes are currently recognized (Jose *et al.*, 2007).

1.9.3.4 Plagues formation on cell culture

Plaque assay in cell culture monolayer are the most common method for quantification of infectious viruses. In these assays, each infectious virus particle multiplies under conditions that results in localized areas of infected cells known as plaques (Mikhail *et al.*, 2006).

Avian influenza viruses replicate in a limited number of cell cultures; Chicken Embryo fibroblast (CEF) are the most commonly used primary cultures whereas the most frequently used continuous cell line is the MDCK.

Few influenza viruses will not grow and produce plaques in cell culture unless trypsin is added to the agar overlay to cleave the HA molecule for production of infectious virus. Using trypsin in the culture medium allows plaques assays with many strains in CEF or MDCK (Calnek *et al* , 1995).

Several plaque tests for influenza viruses have been described (Granoff, 1955; Ledinko, 1955; Gotlieb and Hirst, 1956; Henry and Youngner, 1957; Wright and Sagik, 1958; Choppin,1962; Lehmann - Grube, 1963).

All influenza strains tested were propagated in chick embryos and formed plaques regularly.

Since all influenza virus strain tested formed plaques in primary Chick Kidney Cell (CKC) this system is especially suitable not only for infectivity

test, but also for genetic studies (Babiker and Rott,1968). A wide variety of influenza A viruses comprising human, equine and avian strains grow productively in an established lines of Canine Kidney cells (MDCK) under an overlay medium containing trypsin and formed well defined plaques (Tobital *et al.,* 1975).

Several strains of influenza virus studied for their ability to form plaques in monolayer tissue culture of CEF from 9-11 day old produced plaques in this culture by 4 days (Granoff, 1955).

1.9.3.5 Replication and pathogenesis of the H5N1 virus

In vivo the H5N1 virus enters the host's body either through the respiratory tract or the gastrointestinal tract. In the first instance the host cell would be a cell in the respiratory tract, in the lungs or in the gastrointestinal tract. However the virus can cause systemic dissemination therefore, subsequently, the host cell could be one of many organs, such as the pancreas, kidneys, heart or brain. During the adsorption phase, the HA binds to the host's sialic acid which is a sugar found on cell surface proteins. The HA has mutated which allows it to bind to the different types of sialic acid which occur in humans and birds (Mackenzie, 2006). The cell then engulfs the virion, simultaneously trapping the protease with the virion which attacks the HA. The virus has evolved so that instead of harming the HA, the protease removes a type of 'safety catch' which activates the HA due to post-translational cleavage

of the precursor form of the virus, HA0, by the protease (Alexander *et al*., 2004; Mackenzie, 2006). For low virulence influenza viruses, the HA could only become functional and infectious by being cleaved by proteases found at certain sites, such as trypsin or similar enzymes, which restricted the site of replication of the virus to, for example, the respiratory or digestive tracts. In contrast, HA of highly pathogenic avian influenza (HPAI), can be cleaved by other proteases, such as furin (Stieneke-Grober *et al* ., 1992), which allows the virus to replicate in other parts of the body. This damages vital tissues and organs, causing mortality, which may be as high as 100% (Alexander *et al* ., 2004). It has been found that avian influenza viruses with low virulence have one basic amino acid, arginine, at the HA0 cleavage site whilst HPAI viruses have many basic amino acids, arginine and lysine, next to the cleavage site which can be cleaved by ubiquitous proteases (Senne *et al*., 1996). The host cell pumps in acid to kill the virion however the acid enters through the M2 ion channel triggering a change in the activated HAs. The globular head of the HA folds back and the inside sections bind to the cell membrane (Mackenzie, 2006). This fusion causes the virus capsid to break open and a pore forms between the cell membranes. The RNAs leave the virion during this uncoating process and migrate into the cell's nucleus (Mackenzie, 2006). Polymerase enzymes which were packaged with the RNAs produce messenger RNA (mRNA) copies of viral genes. The host cell then produces thousands of

replications of the ten proteins that were coded for by the viral genome and then copies of viral RNA (Mackenzie, 2006). The new viral surface proteins, HA, NA and the M2 channel, migrate to the cell membrane where the NA destroys any protruding sialic acid which means that the new viral components float to new cells (Mackenzie, 2006). The M1 matrix protein helps to compartmentalise new virions by packing the new viral genome together into cell membranes to join viral surface proteins (Mackenzie, 2006). H5N1 viral replication has been found to be prolonged in human patients with nasopharyngeal isolates being found from a range of 1 to 16 days (median 6.5 days) in 1997 viruses and the onset of illness to first culture ranging from 3 to 16 days (Writing Committee of World Health Organization(WHO,2005). The high frequency of diarrhoea in patients and the detection of viral RNA in faecal samples suggest that viral replication occurs in the gastrointestinal tract (De Jong *et al* ., 2005). This was confirmed by one autopsy (Uiprasertkul *et al* ., 2005).

In vitro influenza virus attachment to the susceptible cell is mediated by the interaction between the viral hemagglutinin and sialic acid receptors present on glycolipids and glycoproteins on the cell surface (Lamb, 1989). At this stage, the sialidase activity of the neuraminidase prevents binding of the HA to sialic acids present in mucopolysaccharadies, which would otherwise interfere with the virus binding to the adequate cellular receptors. The virus is

internalized by endocytosis and, upon acidification of the endosome, conformational changes on the hemagglutinin lead to the fusion between the viral and the endosomal membranes (Lamb, 1989). Acidification of the endosomal lumen also activates the ion channel activity of the viral membrane protein M2 (Pinto *et al .*, 1992). Activation of M2 generates an inward current of protons into the virion's interior that triggers the disassembly of M1 from the vRNPs, which are transported to the nucleus, the site of influenza virus transcription and replication (Martin and Helenius, 1991). A minimal set of four viral proteins is essential for influenza virus transcription and replication: PB1, PB2, PA - referred to as P-proteins-, and the NP protein (Huang, Palese, and Krystal, 1990). Two different populations of positive sense RNAs are synthesized from vRNA templates: messenger RNAs (mRNAs) and complementary RNAs (cRNAs). Viral mRNAs are primed by 5' capped (m7GpppNm-containing) fragments derived from newly synthesized host-cell RNA polymerase II transcripts (Beaton and Krug, 1986; Krug *et al .*, 1989; Plotch *et al .*, 1981; Ulmanen *et al.,* 1983)Viral mRNAs are polyadenylated by a stuttering mechanism involving the viral polymerase and a stretch of uridines, which are located 17-22 nucleotides before the 5' end of the vRNAs (Hay *et al .*, 1977; Robertson *et al .*, 1981); Synthesis of cRNA is the first step in influenza virus replication. Transcription of cRNAs occurs in the absence of primer or polyadenylation and they represent full-length copies of vRNAs

(McGeoch *et al* ., 1976). The second step in viral replication is the synthesis of progeny vRNA molecules from cRNAs templates (McGeoch *et al* ., 1976). Towards the end of the infection cycle and once enough molecules of M1 and NP have been produced, the newly synthesized vRNPs are exported out of the nucleus and assembled into full virus particles. The final assembly steps occur at the plasma membrane exposing the newly synthesized hemagglutinin, neuraminidase proteins, and M2 (Helenius, 1992.) Once the final assembly events are completed, new virus particles bud from the plasma membrane. The activity of the neuraminidase becomes again important by disrupting viral aggregates and thus releasing viral particles that can start a new cycle of infection.

1.10 Prevention and control of the disease

In the face of disease outbreaks in poultry and the potential pandemic threat to humans caused by the highly pathogenic avian influenza viruses (HPAIVs) of H5N1 subtype, improvement in biosecurity and the use of inactivated vaccines are two main options for the control of this disease.(Huoying *et al.* ,2007).

Recent outbreaks of highly pathogenic avian influenza (HPAI) viruses in poultry and their threatening zoonotic consequences emphasize the need for effective control measures. (Van der *et al., 2005*)

Although culling of infected poultry remains the most effective strategy to prevent the transmission of the avian influenza virus, when the viruses are widely spread in multiple domestic and wild avian species such as in the case of the Asian H5N1 epidemic, "stamping out" alone is unlikely to be successful. Depopulation of infected flocks in combination with vaccination of at-risk poultry populations is being implemented in several Asian countries, Italy, and Mexico as an alternative strategy to control the spread of the disease (Capua and Alexander, 2004; Lee *et al*, 2004; Marangon and Capua, 2006).Vaccination of high-risk birds or flocks has been shown to be an effective complementary tool to control the spread of avian influenza (Ellis *et al* , 2004)

1.10.1 Vaccination

When an outbreak of AI occurs in an area with a high population density in which the application of rigorous biosecurity measures is incompatible with the modern rearing systems, vaccination should be considered as a first option to control the spread of infection.

Inactivated vaccines against influenza in birds were used in some outbreaks (Beard, 1991). Vaccination has been prohibited in outbreaks involving highly pathogenic viruses, when eradication is the goal.

The use of genetic engineering has been applied to isolate the haemagglutinin genes, particularly H5 and H7, and place them into alternate

viral vector such as vaccinia virus (Chambers, 1988).These have been used successfully to immunize and protect birds . Swayne *et al(*2000) suggested that vaccination has the potential to reduce environmental contamination with avian influenza virus and prevent subsequent bird-to-bird transmission.

In order to prevent spread of influenza viruses, emphasis must be placed on biosecurity and flock management practices, the development of rapid diagnostics (Lau *et al* , 2004) and vaccine production (Lipatov *et al* , 2004). The vaccination program is a reliable strategy in controlling the disease (Ming *et al* , 2006)

Current influenza vaccines include a subunit vaccine (Babai *et al* , 1999; Laver and Webster , 1976], attenuated vaccine (Horimoto *et al* , 2004; Liu *et al* , 2003), DNA vaccine (Watabe *et al* , 2001) and inactivated influenza vaccine (Cao *et al* , 1992), with the latter being the most widely used on a commercial scale (Lipatov *et al.* , 2004). Vaccination with a commercial H5N2 vaccine proved immunogenic throughout the range of species tested, with some variations between and within taxonomic orders (Joost *et al* , 2007). Vaccination was planned to be used on a nation wide scale in several countries in South East Asia (Normile 2005).

Halvorson (2002) argued that the use of vaccines to control H5 and H7 LPAI infections in commercial poultry decreases the risk of HPAI occurring,

decreases susceptibility to AI infection, decreases the quantity of virus produced, lessens transmission and markedly reduces economic losses. He concluded that there is no justification for not permitting the use of inactivated vaccine to control LPAI (H5/H7) in the absence of HPAI.

Recently vaccines have been developed employing new technologies such as baculovirus derived H5 and H7 haemagglutinins (Crawford *et al* ., 1999) and fowl poxvirus recombinants expressing H7 haemagglutinin (Boyle *et al* ., 2000).

An inactivated H5N2 vaccine was used in Mexico as a result of the widespread HPAI outbreaks caused by H5N2 virus that began in December 1994 (Villareal and Flores 1997). Between the beginning of 1995 and May 1997 847 million doses of vaccine were licensed for use. Inactivated H7N3 vaccine was used extensively in Pakistan following the widespread HPAI outbreaks in 1995 (Naeem, 1998).

Trevor *et al* , 2004 found that use of killed H5N2 vaccine in the face of HPAI H5N1 virus challenge was able to protect chicken from disease and interrupt virus transmission.

1.10.2 Detergents and disinfectants

Influenza viruses are killed by most detergents and disinfectants. Organic material has a negative effect on the efficacy of a disinfectant; viruses which are covered with manure are well protected and can survive up to

approx. 100 days. Complete removal of organic material is therefore an essential part of an effective disinfection procedure (http://www.avian influenza-disinfectant.com/).

Disinfectants active against AIVs can be grouped into soaps and detergents like, alkalis, acids, chlorine and chlorine compounds, oxidizing agents, aldehydes, phenol compounds, quaternary ammonium compounds (QACs) and alcohols (Maris, 1995; Ausvetplan, 2005).

The influenza viruses are susceptible to a variety of disinfectants including sodium hypochlorite, 70% ethanol, oxidizing agents, quaternary ammonium compounds, aldehydes (formalin, glutaraldehyde, formaldehyde), phenols, acids, povidone-iodine and lipid solvents(Center,2007).

phenolic disinfectants, a quaternary ammonia compound a peroxygen compound,Virkon-S and sodium hypochlorite (household bleach). All five disinfectants were effective at inactivating AIV at the recommended concentrations (Suarez *et al.*, 2003)

1.11 Avian influenza in Sudan

The disease was reported in the Sudan for the first time in 1923 (Report of the Sudan Veterinary Service, 1923) Since then Elmubark (1970) reported that he was unable to isolate the virus of fowl plague. Then Elamin and Khier (1985) using the AGID to detected antibodies to influenza virus in large animal

sera in Kassala region, they reported that positive reaction for influenza virus type A were obtained in two (4.7%) of 42 camel sera, four (5.4%) of 74 goat sera, six (7.2%) of 86 sheep sera, two (2.9%) of 70 cattle sera and no antibodies were detected in donkey sera.

The first report of isolation of avian influenza virus was done by Elamin (2000) from outbreaks in poultry farm in 1998 in Khartoum State; she was able to obtain 31 isolates from 97 samples.

In 2006 Eltahir found that 24% out of 100 human sera and 32% out of 100 chicken sera were positive by using AGID. Saeed (2006) used the hemaggltination inhibition test against H7 and H5 antigens for sera collected from 100 chicken and 100 human, the results show that 21 out of 100 collected from chicken were positive with antibodies against H7 antigens, and two were positive against H5 antigens, also 15 sera were positive out of 100 collected from human.

AI H5N1 was reported in Sudan for the first time in April 2006 in two poultry farms in Khartoum and Aljazeera State (OIE, 2006).

Wegdan and Kheir (2007) were able to isolate a HPAI H5N1 from outbreak which occurred in six farms in Khartoum and two farms from AlGazeera States in 2006.

1.12Avian influenza in humans

Avian influenza viruses also pose a significant threat to human health as these viruses have been shown to be able to infect humans. AI virus infection is not usually considered a zoonotic infection, but under certain circumstances, the virus poses a serious public health threat (Chang et al., 2005). The two subtypes, namely H5 and H9, that are currently endemic in poultry in some regions of the world, have been shown to be capable infecting humans (Subbarao *et al .,* 1998; Bridges *et al .,* 2002) and there have been several reports of H7 viruses infecting humans, including those isolated during the recent HPAI outbreaks in the Netherlands and Canada (Alexander, 2006).

In 1996 an H7N7 virus was isolated in England from the eye of a woman with conjunctivitis who kept ducks. This virus was shown to be genetically closest in all eight genes to viruses of avian origin and nucleotide homology in the HA gene with a virus of H7N7 subtype isolated from turkeys in Ireland in 1995 (Kurtz *et al .,* 1996).

In May 1997, a highly virulent strain of influenza A (H5N1) virus entered the human population, causing a fatal illness in a 3-year-old boy living in Hong Kong (De Jong *et al* , 1997). This strain of influenza virus received worldwide attention when 17 additional cases with 5 deaths were described

during the following winter. This virus has features common to other highly pathogenic avian viruses, including a series of basic amino acids adjacent to the cleavage site of the hemagglutinin (HA) (Subbarao *et al* , 1998; Claas *et al* , 1998).

There was some evidence of very limited human to human spread of this virus, but clearly the efficiency of transmission was extremely low (Buxton Bridges *et al* ., 2000). There have been no new cases since December 1997. The viruses isolated from the human cases appeared to be identical to viruses first isolated from chickens in Hong Kong in March 1997 following an outbreak of highly pathogenic disease. Both human and avian isolates possess multiple basic amino acids at the HA0 cleavage site.

It has been suggested that a pandemic of influenza could begin with an isolated case of an avian or swine influenza virus strain crossing the species barrier and adapting to the human host, preceding rapid spread of the virus (Webster *et al*, 1997).

The first outbreak of human cases of avian influenza was reported in 1997 in Hong Kong. Since 2003, there have been many small outbreaks of human cases around the world, and the reported mortality is greater than 50%. Current evidence suggests that the human-to-human transmission of avian

influenza is rather inefficient, but mutation might occur in the future resulting in improved transmission and possibly a pandemic in human (Wong and Leung , 2007).

CHAPTER TWO

MATERIALS AND METHODS

2.1. Study area

The study was conducted in Khartoum State because it has the largest chicken population in the country.

2.2. Samples

Seven hundred and sixty six samples consisting of 200 tracheal, cloacal swabs, 50 tissues (lung, trachea, liver, spleen and intestine content) and 516 serum samples were collected from White Bovan chickens in different farms in Khartoum area (Table 1).

2.2.1. Collection of samples

2.2.1.1 Swabs and tissues

Tracheal and cloacal swabs were collected from dead and live chickens by using absorbent cotton swabs, labeled and replaced in containers. Samples from lungs, spleen, liver, trachea and heart were also collected aseptically by sterile scissors and forceps, placed into sterile bottles containing Phosphate Buffer Saline (PBS) with 5% antibiotics (Penicillin, Streptomycin, Gentamycin, and Mycostatin) and labeled. All samples were transported to the laboratory on ice and then stored at - 40ºC until used.

Table 1: Geographical sites where the samples were collected

Farms	Population Of chicken	Chicken age in weeks	Samples		
			T.S	C.S	T
Shambat	3000	48	30	30	-
Alhalfaia	2000	8	20	20	30
Algirif West	2000	8	20	20	20
Alfaki hashem	10000	18	-	10	-
Alftahab	2000	12	30	20	-

T.S = Tracheal Swab.

C.S = Cloacal Swab.

T = Tissue (trachea, lung, liver, spleen and heart)

2.2.1.2. Serum samples.

The blood was collected from chickens in commercial farms and government farms in Khartoum State.

Blood samples were taken from the wing and right jugular vein into sterile Bijou bottles and were left to clot at room temperature. The clot was separated from the edges of the bottles and left overnight at 4 ºC. Sera were taken then collected and stored at -20 ºC until used

2.3. Embryonated hen's eggs

One day old fertile eggs were obtained from a flock of White Bovan chickens in the department of Preventive Medicine, Faculty of Veterinary Medicine and from Koral commercial poultry farm, Khartoum. The eggs were incubated at 37 ºC in an egg incubator and were turned daily for ten days and used as eleven day- old embryo. Embryonated eggs were also obtained from CVRL farm, Soba at 9-10 days of age.

2.4 Sterilization

2.4.1. Glassware and instruments.

Glassware like beakers, flasks, pipettes, centrifuged tube, bottles, measuring cylinder were rinsed in tap water and all solid matter washed away. They were then immersed overnight in 1% NaOH , after which they were immersed in 1% Hcl for two hours the following day and then boiled in water

with detergent for 20 minutes and rinsed in running tap water for five times to remove detergent completely. After that they were rinsed in 5 changes of distilled water, left to dry and finally sterilized in the oven at 180^0C for 2 hours. Scalpels, scissors, forceps, pestle and mortar were sterilized in the hot air oven at 160 ºC for 2 hours.

2.4.2 Sterilization of microtiter plates

When live virus was used the plates were shaken in a solution of 1% NaoH till the red cells were loosened and removed, they were then soaked in the same solution overnight, and in the following day they were rinsed in tap water and soaked in hydrochloric acid solution for two hours. They were then rinsed in four changes of deionized distilled water (DDW) and left to dry at room temperature.

2.4.3 Solutions and plastic ware.

Saline, buffer Solutions, sand, measuring cylinder and bottles with rubber caps were autoclaved at 121 ºC for 15 minutes.

2.5 Avian influenza antisera.

Positive sera for H5N3 and H7N1 were obtained from Central Veterinary laboratory, Soba.

2.6 Avian influenza antigens.

Reference strains H5N3, H7N1 for hemagglutination inhibition test and H5N2 for Agar Gel Immunodiffusion Test were obtained from Central Veterinary laboratory, Soba.. Inactivated reference H5N1 type antigen was obtained from a commercial source in Khartoum.

AI antigen for agar gel Immunodiffusion test (AGID) was also prepared in eleven-day-old chorioallantoic membrane (CAM) of 11-day-old chick embryos from field isolates.

2.7 Isolation of virus

2.7.1. Preparation of inoculum

Swabs were rotated for one minute and the fluids were then centrifuged at 1000 r p m for 5 minutes. The supernatant was collected in sterile containers and stored at -40 ºC until used.

In case of tissues (lung , liver , spleen , heart) , 10% suspensions in PBS were prepared by pooling half gram of each tissue and mincing with sterile scissor and grinding with sterile mortar and pestle using sterile sand. The mixture was then added to 10 ml PBS containing antibiotics .Then the latter was centrifuged at 1000rp m for 5 minutes. The supernatant was

carefully removed and stored at – 40ºC until used for egg inoculation (OIE Manual, 1996).

The supernatant was then aseptically removed and placed in vials for egg inoculation and storage. Specimens were kept at room temperature with antibiotics for 1- 2 hr before inoculation into eggs to reduce bacterial contamination.

2.7.2. Egg inoculation

Five 9-11- day – old embryonated chicken eggs were used for each sample and other five embryonated eggs were kept as un inoculated control.

Before inoculation all embryos were candled for viability, and the site of inoculation was marked which must be free of large blood vessels, and disinfected with 70 % alcohol. A hole was then made in the labeled area between the air sac and the CAM by blunt needle. O.2 ml of the inoculum per egg was introduced into the allantoic cavity by inserting the needle 5/8 inch vertically through the hole away from the center of the egg. The inoculated eggs were rocked gently to ensure even distribution of the inoculum. Then the hole was sealed with wax and the inoculated eggs were incubated in a humidified atmosphere at 37ºC without turning for four to seven days.

2.7.3. Examination of inoculated eggs.

Inoculated eggs were candled once a day and embryos that died within 24 hours were discarded as non specific deaths due to injury or bacterial contamination. All embryo deaths beyond 24 hr were attributed to the growth of the virus. The dead and live inoculated eggs were refrigerated for at least four hours at 4°C before harvesting.

2.7.4. Harvesting and preservation of allantoic and amniotic fluids and CAM.

The shell over the air sac was disinfected with 70% alcohol and cracked by tapping with the blunt end of sterile forceps. The CAM, and the amniotic membrane were gently ruptured using sterile forceps ,the fluids were aspirated with a sterile syringe and clarified by centrifugation at 1000 r p m for 10 minutes and the supernatant was placed into sterile eppendorf tubes and kept at – 20ºC until used .

The CAM was collected after discarding the contents of the eggs with sterile forceps and placed in a Petri dish containing sterile normal saline. It was examined for virus growth, thickening of CAM and hemorrhage, then collected in sterile containers and kept at -20 ºC.

2.7.5. Passage of isolates.

The harvested fluid was centrifuged at 1000rp m for 5 minutes. 0.2 ml of the supernatant containing 5 % antibiotics was inoculated into the allantoic sac of 10-day old embryonated eggs.

2.8 Identification of virus.

This was based on:

2.8.1. Death of embryos.

Inoculated eggs were examined by candling to detect embryos mortality. Embryos usually died 36 to 96 hours after inoculation.

2.8.2. Hemagglutination test for chicken RBCs .

2.8.2.1. Preparation of 1% chicken RBCs .

This was done according to OIE manual,1996

2.8.3 Hemagglutination test (HA).

This test was carried out as described by (OIE Terrestrial Manual, 2008).

2.8.4 Hemagglutination inhibition test (HI)

This test was performed as described by (OIE Manual, 1996).

2.8.5 Agar gel precipitation test (AGPT) for antigens.

2.8.5.1 Preparation of antigen.

Allantoic fluid from passage two of the field isolates were inoculated in amount of 0.2 ml in embryonated eggs. Five eggs were used for each isolate.

The five isolates were A-1, A-11, A-45, C-1 and C-45.The CAM and the allantoic and amniotic fluids were collected under aseptic conditions and kept frozen at -20 0C until used in HA. The CAM antigen was ground in 5 ml PBS containing 5% antibiotics in a mortar, then homogenized in a homogenizer and kept at -40ºC.The antigen was frozen and thawed three times before being used in AGID.

2.8.5.2 Preparation of agar gel

This was prepared by dissolving one gram of agarose in 100 ml of normal saline 0,85%.0.5 mg of sodium azide was added to all agar preparations as a preservative. The agar was boiled for 45-60 minutes it was then distributed in 0.5 ml amount in Petri dishes, placed on the bench at room temperature to solidify and then kept at 4ºC.

2.8.5.3 Testing and examining of agar plates

A rosette of six peripheral wells and a central well were cut with a template. The plugs were carefully removed with a needle. Each peripheral well was filled with 25 microliter of antigen using microliter pipette while the central well was filled with 25 microliter of reference antisera. The plates were incubated in a humid chamber for ten days at room temperature and were examined daily in a dark room through transmitted light for precipitation bands.

2.9 Virus isolation and plaque production in cell culture

2.9.1. Chick embryo fibroblast monolayer culture (CEF).

A group of 9-10 day-old embryonated eggs were used after being candled to check for viability. The blunt end of the eggs was sterilized by swabbing with 70% alcohol. The shell over the air sac and the membrane were peeled off and the CAM was broken. The embryos were hooked around the neck and transferred to a Petri dish containing 10 ml of cold PD. The embryos were then drained and transferred to a dry Petri dish .The head, viscera and limbs were discarded. The bodies were minced with a pair of sterile scissors. The minced bodies were transferred to a trypsinization flask and washed twice with PD solution by gentle stirring with magnetic stirrer for one minute. Trypsinization was carried out each time for one minute. The digested cells were poured into a flask on ice containing 1-5 ml calf serum to stop the action

of the remaining trypsin. Then the cells suspension was filtered through a muzlin gauze and centrifuged for 10 minutes at 1000 rpm in refrigerated centrifuge. The cell pellet was broken up by gentle repeated pippetting in 10 ml of growth medium to break up cell clumps. The resultant cells were resuspended in 100 ml of growth medium after measuring the PCV.

2.9. 2 Chick Kidney Cell Culture (CKC).

5-6 day-old healthy chicks were used. The chicks were scarified and dipped in 70% alcohol. The abdomen was opened and the kidneys were displayed. They were aseptically removed and washed in PD three times. The fibrous tissue was picked out and the kidneys were chopped with a pair of scissors and washed once more in PD solution. The tissue fragments were trypsinized repeatedly using 0.25% trypsin solution in PD at intervals of 10 minutes until they were completely digested. The suspension was then passed through a muzlin funnel and washed three times by centrifugation at the rate of 1500 rpm for 10 minutes. They were finally suspended in outgrowth medium and dispensed in small flasks.

2.9.3 Inoculation of the virus isolates in CEF monolayer and CKC

The established monolayer should be confluent and non should show vaculation. The growth medium was decanted and the sheets were washed twice with sterile PBS. Serial 10-fold virus isolate dilutions from 10^{-5} -10^{-10} were each inoculated in a pair of dishes at the rate of 0.2 ml per well of tissue

culture plate and flask and incubated at 37°C for 30-45 minutes for plaque production. During this time the first overlay medium mixture was prepared and incubated at 42°C.

2.9.4 Cytopathic effect of the isolates

For demonstration of cytopathic effect (CPE), CEF and CKC monolayer were prepared in 25 ml plastic cell culture flasks that had cover slips put inside. The growth medium was discarded and all flasks were washed with sterile PBS. 1 ml volume of 10^{-5} dilution of the isolate was inoculated in two flasks. All flasks were incubated for 45 minutes at 37°C for virus adsorption, then 10 ml of outgrowth medium was added to each flask and all were incubated for 24 hours at 37°C and the presence of CPE was observed daily.

2.9.5 Addition of overlay medium

The overlay medium was added in the rate of 3 ml/plate per well and left for 10-15 minutes to harden and was then transferred to CO_2 incubator, preferably after sealing, incubated at 37°C for 4 days during which the plates were observed daily for presence of plaques (Appendix IV).

2.10 Serological methods

2.10.1 Chicken sera for serological tests

These bloods were collected from different farms in Khartoum State.

2.10.2 Hemagglutination test (HA) for chicken RBCs

2.10.2.1 Preparation of 1% chicken RBCs.

An equal volume of Alsever's solution and chicken blood obtained from the wing vein of disease controlled chickens, were collected in sterile syringes, mixed gently and transferred slowly to a large centrifuge tube for washing. An equal amount of PBS at pH 7.2-7.4 was added and the suspension was centrifuged at 500 r p m for 5 minutes , the supernatant was poured off , and 20 volumes of PBS was added to the packed cells , the centrifugation step was repeated three times . The cells were then used to prepare 1 % suspension based on volume by adding 1 ml of the packed cell to 99 ml of PBS and was stored at 4 ºC until used.

2.10.2.2. Performance of Hemagglutination test (HA) for chicken RBCs

HA was performed according to OIE manual (1996). Twenty five microtiter (µl) of PBS was dispensed into each well of a plastic microtiter plate, 25 µl of allantoic fluids was placed in the first well. Two-fold dilution was done. Then 25 µl of 1 % chicken RBCs was dispensed to each well, the plate was tapped gently and then the RBCs were allowed to settle for 40 min at room temperature.

2.10.3 Hemagglutination inhibition test (HI).

HA test was made on undiluted influenza antigens. The last well that give complete HA was considered one HA unit and the previous well considered the 2HA,the 4HA unit were accurately calculated. The virus suspension was then diluted to contain 4HA unit per 25µl

Twenty five microtiter (µl) of PBS were added to all wells in plastic microtiter plate, 25 µl of tested sera were added into well one, then two-fold dilution were performed. The diluted serum samples were mixed with 4HA units of virus and incubated at room temperature for 30 min. Then 25 µl of 1% chicken RBCs was added to each well and plates were incubated at room temperature for 30-45 min. The HI titer was expressed as the reciprocal of the highest serum dilution that completely inhibited hemagglutination of 4HA units of the virus.

2.10.4 Agar gel precipitation test (AGPT) for antibody detection

2.10.4.1. Preparation of agar gel

This was applied according to (Beard, 1970).

2.10.4.2. Testing and examination of plates.

The plugs were carefully removed with a needle. Each peripheral well was filled with 25 microtiter of tested sera using microtiter pipette while the central well was filled with 25 microtiter of reference antigen.The plates were incubated in a humid chamber for ten days at room temperature and were examined daily in a dark room through transmitted light for precipitation bands.

2.10.5 Enzyme- Linked Immunosorbent Assay (ELISA) for the detection of antibodies of avian influenza viruses.

The AI ELISA Kit was used for detection of antibodies of avian influenza viruses type A (BioChek B.V. Crabethstraat 38-C 2801 AN Gouda Holland).

2.10.5.1 Reagents and solutions supplied in the kit

- Five coated microtiter plates with inactivated AI antigen
- Negative control serum (1 vial 3 ml)
- Positive control serum (1 vial 3 ml)
- Conjugate reagent (Sheep anti chicken 1 vial)
- Substrate tablets (12 tablets)

- Substrate Buffer(1 vial 55 ml)
- Stop solution (1 vial 55 ml)
- Sample Diluent (1 vial 250 ml)
- Washing buffer (Phosphate Buffer Saline with Tween)

2.10.5.2 Reagents preparation

According to manufacture of the kit instructions

2.10.5.2.1 Preparation of substrate reagent

Two tablets were added to 11 ml of substrate buffer and allowed to dissolve for 3 minutes before being used.

2.10.5.2.2 Preparation of washing buffer

The contents of the washing buffer sachet were dissolved in one litre of distilled water.

2.10.5.3 Dilution of samples

The tested sera were diluted 1:500 by adding 1µl to 500 µl of sample diluents and then mixed well by shaking the tubes.

2.10.5.4 Test procedure

The kit was removed from refrigerator and put at room temperature before use. The coated plates were removed from the sealed bags.

- 100µl of negative control serum were added into wells A1 and B1.

- 100 µl of positive control serum were added into wells C1 and D1.

- 100 µl of diluted tested sera were added into the appropriate wells, and then plates were covered and incubated at room temperature for 30 minutes.

After incubation the plates were removed and the contents of wells were discarded by flicking them into a sink. All wells were then filled with the washing buffer, shaking gently and emptied into a sink. The washing step was repeated three times, and then the plates were tapped on absorbent paper.

2.10.5.4.1 Addition of the conjugate

Amounts of 100 µl were added to each well. The plate was then shaken gently and incubated at room temperature for 30 minutes. After incubation the plates were removed and the contents of wells were discarded and then the step of washing was repeated.

2.10.5.4.2 Addition of substrate

Hundred microlitre of the substrate buffer was added to each well. The plate was left at room temperature for 15 minutes.

2.10.5.4.3 Addition of stopping solution

After 15 minutes 100 µl was added to each well to stop the reaction.

2.10.5.4.4 Reading of the plate

Using the ELISA reader the plate was read at 405 nm and the result was printed out.

2.10.5.4.5 Calculation

Using sample/positive ratio(S/P ratio) which is calculated as follows:

$$S/P = \frac{\text{Mean of test sera- Mean of negative control}}{\text{Mean of positive control- Mean of negative control}}$$

2.11 Biological properties of the viruses

2.11.1 Hemagglutination of avian and mammalian erythrocytes

2.11.1.1 Preparation of 0.75 % horse RBCs.

One ml blood was collected into clean sterile tubes containing 2 mg sodium citrate, mixed thoroughly to prevent clotting, centrifuged at 200 rpm

for 5 minutes, the supernatant was discarded and cells were resuspended in normal saline to make a 0.75 % suspension for agglutination test in plates.

2.11.2 Determination of the Minimum Lethal Dose50 (MLD_{50})

The MLD_{50} was determined. A total of 30 9-11-day-old embryonated eggs were used for inoculation of each isolate.10 fold serial dilutions starting from 10^{-1} throughout to 10^{-12} were prepared from freshly harvested allantoic fluids from the second passage of each virus isolate. Sterile PBS containing antibiotics was used as diluent. Five embryonated eggs were inoculated in the allantoic cavity with each dilution starting from 10^{-1}.Control eggs were inoculated with diluent only. The MLD_{50} was calculated by the methods of Reed and Muench (1938).

2.11.3 Thermal inactivation

Freshly harvested allantoic fluids from the fourth egg passage were pooled and divided into 10 portions. One was frozen at – 40ºC. The others were placed in 50 ml screw capped tubes and submerged in a water bath at 37, 56 and 70ºC. The contents of the tubes were mixed from time to time before each sampling. Samples were collected 5, 10, 15, 30 and 60 minutes after the start of the experiment and then rabidly chilled in ice and a portion of each

sample was immediately frozen. A portion of each one was used for an immediate HA titration.

2.12 Resistance to disinfectants

Three isolates A-11, A-45 and C-45 were used after three passages. Infected allantoic fluids (AF) diluted 1:2 in PBS were mixed with disinfectants at the ratio of 1:1. The mixtures were held at room temperature as indicated in table (3). Disinfectants used were 2% Phenol, 8%Formaline, 70%Ethanol, 1% Verkon (s) and 2% Gluteraldahyde.

2.13 Molecular Technique

2.13.1 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reverse transcription polymerase chain reaction was used for examining positive specimens for confirmation.

2.13.1.1 Viruses

Five virus isolates of avian influenza, isolated from chickens in Khartoum State were propagated in embryonated eggs to obtained fresh allantoic fluids.

2.13.1.2 Primers

Two primers were used for H5 gene. (IAEA Laboratory, Seibersdorf ,Vienna)

H5-kha-1: 5′-CCT CCA GAR TAT GCM TAY AAA ATT GTC- 3′

H5-kha-3 : 5′- TAC CAA CCG TCT ACC ATK CCY TG - 3

2.13.1.3 Extraction method of AI RNA from allantoic fluid

Freshly collected allantoic fluids, containing virus in a concentration of not less than 10^5 MLD_{50} per 0.1 ml, was used.

The allantoic fluids was brought out and allowed to thaw. The samples were put into 1.5 ml eppendorf tube at the rate of 0.1ml/tube.To each tube, 1000 µl of Trizol was added; tubes were tightly closed, mixed gently and kept for 5 min at room temperature. Then 200µl of chloroforml were added, the tubes were mixed gently by inverting it up and down. Tubes were then centrifuged at 10 000 rpm for 15 min at 4ºC.After centrifugation, three layers were formed of which the colorless upper aqueous layer containing the RNA. The upper layer that contained the RNA was transferred to a new 1.5ml tubes and to this volume of 500µl isopropanol was added and mixed by inverting the tubes up and down. The tubes were incubated overnight at -20ºC.Tubes were then centrifuged at 10 000 rpm for 20 min at 4ºC.After centrifugation, RNA precipitated at the side of the tube. Supernatant was removed using a micro pipette. To each 1.5 ml tube that contains the RNA pellet,500µl of 75% ethanol alcohol were added for washing, gently mixed and centrifuged at 12 000 rpm for 10 min at 4ºC.After centrifugation the RNA pellet was carefully dried by

placing the tubes up side down on a clean paper for 5-10 min at room temperature. After drying, 50µl of RNase free water (water for injection) were added to every tube, mixed well to dissolve the RNA pellet. The final concentration and purity of each RNA sample was measured using spectrophotometer and the sample quality was monitored by loading a mixture of 2µl RNA, 8µl distilled water and 2µl loading buffer into a 2 % agarose gel at 50 volts for half an hour. RNA marker was also loaded.

2.13.1.4 Determination of the RNA concentration and purity

The concentration and purity of the extracted RNA were determined by the nano-drop instrument at wavelength of 260/280. The pure preparation of RNA has a ratio 1.6 - 1.9. The RNA stock was stored at – 20ºC until used.

2.13.1.5 Preparation of the Master Mix

The RT-PCR was carried out in a master mix mixture (25µl) containing 2.5 µl of 10-times reaction buffer, 2.5 µl dNTPs, 1 µl reverse transcriptase (200 units/µl), 0.3 µl RNase inhibitor (40 units/µl), 1.25µl $MgCl_2$, 0.5 µl Taq DNA polymerase (9 units/µl), 1 µl of each primer (10 pmol each), and 15 µl of water.

2.13.1.6 Thermocycling

The PCR condition was done 42ºC for 45 min (reverse transcription),95ºC for 3 min,35 cycles of 95ºC for 30 seconds (denaturation),

50ºC for 40 seconds (annealing) and 72ºC for 40 seconds (extension), followed by 72ºC for 10 min (final extension).

2.13.1.7 Preparation of agarose gel for electrophoresis

2% agarose gel electrophoresis was prepared by dissolving 1gm agarose in 45 ml distilled water and 5 ml of 1X TBE buffer. The agar was dissolved by heating. The melted agar was Cooled to 60ºC, and then 3µl of ethidium bromide was added and mixed well. The melted agarose was poured into the gel-casting tray. It was allowed to solidify at room temperature. The gel casting tray was placed onto a gel casting base. The tray was placed into the electrophoresis chamber with the wells at the cathode side. The buffer chamber was filled with 1X TBE at a level that can cover the top of the gel. For sample loading, 5µl gel loading buffer was added to each 10µl PCR products.

5µl of the molecular weight marker (50 bp DNA ladder, promega) were mixed with 10µl volume loading buffer then the mixture was loaded into the first well of the agarose gel. 15µl of the PCR product was Pipetted to the gel. The lid was closed on chamber and attached to the electrodes. The gel was run at 50 Volts for 1.4 hours.

2.13.1.8 Visualization of the gel

The gel was visualized under ultra violet transilluminator and photographed in a gel documentation system. The size of PCR fragments were stimated from their relative distance of migration to the molecular weight marker.

CHAPTER THREE

RESULTS

3.1 Field observations

Many poultry farms were visited, where some birds showed respiratory signs, but no outbreak of the disease occurred during the study period until 2006 when the first outbreak of the AI was reported in a poultry farm in Khartoum North area. The birds showed typical sings of the disease which included depression, ruffled feathers, swelling of the face and head, cyanosis of the comb and wattles, diarrhea and respiratory signs including sinusitis and nasal discharge.

3.2 Isolation and identification of the virus

3.2.1 Isolation of the virus in embryonated eggs

A total of 5 isolates (2%) were recovered from 250 samples, 3 (3%) isolates from 100 cloacal swabs, one (2%) isolate from50 tissue samples (liver, lung, trachea) and one (1%) isolate from100 tracheal swabs (Table 2). All the isolates were obtained from Khartoum North area.

The isolates caused death of the embryos within 48 to 96 hr as shown by candling.

3.2.2 Identification of the virus by Agar gel precipitation test

3.2.2.1 Reference avian influenza antisera against infected CAM

Precipitation bands were formed between the reference antisera and all positive sample antigen .The bands were slightly faint. Negative CAM showed no precipitation band.

3.2.3 Haemagglutination of chicken and horse RBCs

All isolates agglutinated Chicken RBCs indicating that they were probably influenza viruses. They also agglutinated horse RBCs .

3.2.4 Hemagglutination inhibition test

The test was done using Newcastle disease antisera against the isolates to exclude the possibility that hemagglutination was due to Newcastle disease virus. After that the allantoic fluids negative for Newcastle disease were tested against reference avian influenza antisera

Table 2: Isolation of influenza virus in embryonated eggs

samples	**Number examined**	**No. positive**	**No. negative**
Cloacal swabs	100	3 (3 %)	97
Tracheal swabs	100	1 (1 %)	99
Tissues	50	1 (2 %)	49
Total	250	5	245

3.3 Prevalence of AI in Khartoum State as tested by

3.3.1 Agar gel precipitation test of sera obtained from the farms

Five hundred and sixteen sera ,254 before the outbreak and 262 after the outbreak from different farms in Khartoum State were tested by AGID against reference antigen H5N2.

The results showed that 23 out of 254 sera were positive 19 out of 66 sera were positive from Khartoum farm 1 and 4 out of 74 were positive from Soba farm 2 before the outbreak and all sera from Kalakla,Fitaihab , halfaia and Shambat were negative (Table 3). Regarding sera collected after the outbreak in 2007, 11 (4.2%) out of 262 sera were positive from Fakihashim farm and all sera from other farms were negative (Table 4).

3.3.2 Enzyme –linked immunsorbant assay (ELISA)

3.3.2.1 Detection of antibodies in tested sera

The 254 sera collected during 2004-2005 and 262 collected in 2007 were tested for presence of avian influenza antibodies using the ELISA test for type A. Positive and negative sera were determined using the S/P ratio.

Sera reading 0.500 or greater were considered as positive while sera reading 0.499 or less were considered as negative.

The results showed that 94 (37%) were positive from 2004-2005, 2 out of 25 were positive from Fitaihab farm, 13 out of 40 were positive from Halfaia farm, 3 out of 24 were positive from Shambat farm, 58 out of 66 were

positive from Khartoum farm 1 and 18 out of 74 were positive from Soba farm 2 (Table5).The result also showed that 41(16%) were positive from sera collected in 2007 after the outbreak (Table 6).

3.3.3 Haemagglutination inhibition test

Positive sera obtained by ELISA were tested against two reference antisera H5N3 and H7N1.Atotal of 94 sera collected before outbreak and positive to ELISA were tested by HI test, the results obtained was that 28 sera were positive, 17(60.7%) for H5N3 and 11(39.3%) for H7N1. The results for sera collected after the outbreak showed that only 6(14.6%) sera out of 41 positive to ELISA were found positive to H5N3 (Table 7).

3.4 Pathogenicity test of isolates

3.4.1 Growth on cell culture and plaque production

The isolates produced large plaques with high efficiency in CEF. They were also plaqued in CKC, with more varied shapes and efficiency was slightly reduced.

Results in CKC were better. Five isolates of avian influenza viruses were tested for the ability of these isolates to form plaques on cells cultures. One isolate readily produce countable plaques (Figure 5).Thus, CEF and CKC appear suitable for plaque assaying of AI viruses.

The isolates were found to be Cytopathic for CKC and CEF. The CPE was first observed after 24 hours post infection, it was similar for all inoculated

isolates. Figure (1) showed normal tissue culture of CEF, figure (3) showed normal tissue culture of CKC and figures (2 and 4) showed the CPE of the isolates.Clear CPE was observed for all isolates tested.

3.5 Biological properties of the isolates

3.5.1 The Minimum Lethal Dose (MLD_{50})

Five viruses were isolated during the study from 250 samples. Titration of the viruses in ten-day-old chick embryos was done. The chick-embryo 50% leathality rate (MLD_{50}) for the five isolates were calculated by Reed and Muench method (1938) these were shown in (Table 8).

3.5.2 Thermal inactivation

The results of this study confirmed that avian influenza virus is readily inactivated by heat. Inactivation experiments were conducted as previously described. Inactivation levels were determined by comparing virus titers. (Tables 9, 10)

3.6 Resistance to disinfectants

The three isolates A-11, A-45 and C-45 proved to be resistant to 2% phenol at one min, but were completely inactivated at 30min. 2% Gluteraldahyde was also found to be ineffective at one min and 10min. 8%Formalin caused an almost complete inactivation of the viruses at all exposure times. 1%Virkon$^{(s)}$ and 70% ethanol reduced the virus titer in 30 min (Table 11).

3.7 RT-PCR

3.7.1 Purity and concentration of RNA extracts

For extraction of viral RNA from allantoic fluid, the Trizol method was used (Ming, 2001),when the viruses were exposed to 1 ml Trizol for 5 minutes at room temperature they gave purity range from 0.81-1.6 and concentration range from 29.4-89.1 ng/µl (Table 12).

3.7.2 Confirmation of the virus isolates by RT-PCR

Two primers were used to detect, identify and confirm the isolates. Five isolates were tested by RT-PCR. All 5 isolates collected from one farm in Khartoum north area in 2006 were found positive after RT-PCR amplification using H5 gene primers of AI virus (Figure 6).

All of them gave PCR fragments of about 300 base pairs (bp) on 2% agarose gel when estimated from their relative distance of migration of the molecular weight marker (Figure 7). The results were confirmatory for the virus isolation and serological sub typing.

The isolates were found to be AI virus type A subtype H5 when compared to the positive control.

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Table (3): Results of AGID for sera collected before AI outbreak in Khartoum State 2006-2007

Locality	AGID		Total
	POSITIVE	NEGATIVE	
Kalakla	-	25	25
Fitaihab	-	25	25
Halfaia	-	40	40
Shambat	-	24	24
Farm1 K	19	47	66
Farm2 S	4	70	74
Total	23 (9%)	231	254

K Khartom

S Soba

Table (4): Results of AGID for sera collected after AI outbreak in Khartoum State 2006-2007

Locality	AGID		Total
	POSITIVE	NEGATIVE	
Halfaia	-	35	35
Koko	-	25	25
Shambat	-	13	13
Fakihashim	11	44	55
Drwshab	-	35	35
Fitaihab	-	16	16
Samrab	-	38	38
Tebna	-	45	45
Total	11 (4.2%)	251	262

Table (5): Detection of antibodies using ELISA test for sera collected before AI outbreak in Khartoum State 2006-2007

Locality	ELISA		Total
	POSITIVE	NEGATIVE	
Kalakla	-	25	25
Fitaihab	2	23	25
Halfaia	13	27	40
Shambat	3	21	24
Farm 1K	58	8	66
Farm 2S	18	56	74
Total	94 (37%)	160	254

K Khartom

S Soba

Table (6): Detection of antibodies using ELISA test for sera collected after AI outbreak in Khartoum State

Locality	ELISA		Total
	POSITIVE	NEGATIVE	
Halfaia	6	29	35
Koko	2	23	25
Shambat	2	11	13
Fakihashim	19	36	55
Drwshab	4	31	35
Fitaihab	2	14	16
Samrab	-	38	38
Tebna	6	39	45
Total	41 (15%)	221	262

Table 7: Comparison of positive results of sera tested with ELISA, AGID and HI test

outbreak	Total of samples	ELISA	AGID	HI		subtype
				positive	negative	
Before	254	94	23	28	66	H5N3 17(60.7%) H7N1 11(39.3%)
after	262	41	11	6	35	H5N3 6 (100%) H7N1 0

Table (8): Determination of the Minimum Lethal Dose (MLD_{50}) of AI iso;ates

Virus	MLD_{50}
A-1	$10^{5.6}$
A-11	$10^{7.4}$
A-45	$10^{6.6}$
C-1	$10^{5.5}$
C-45	$10^{5.6}$

Table 9: Inactivation of the isolates by heat

		Time (min)					
Isolate	Temperature	0	5	10	15	30	60
A-11	37 ^{0}C	+	+	+	+	+	+
	56 ^{0}C	+	+	+	+	-	-
	70 ^{0}C	+	-	-	-	-	-
A-45	37 ^{0}C	+	+	+	+	+	+
	56 ^{0}C	+	+	+	+	-	-
	70 ^{0}C	+	-	-	-	-	-
C-45	37 ^{0}C	+	+	+	+	+	+
	56 ^{0}C	+	+	+	+	-	-
	70 ^{0}C	+	-	-	-	-	-

+ Positive for HA

- Negative for HA

Table (10): Effect of temperature on the stability of haemagglutinin of three influenza virus isolates

Time / Isolate	Temperatures																	
	Titer																	
	37ºC						56ºC						70ºC					
	5	10	15	20	30	60	5	10	15	20	30	60	5	10	15	20	30	60
A-11	160	160	160	160	160	160	80	80	80	80	0	0	0	0	0	0	0	0
A-45	160	160	160	160	160	160	80	80	80	80	0	0	0	0	0	0	0	0
C-45	160	160	160	160	160	160	80	80	80	80	0	0	0	0	0	0	0	0

Table 11: Inactivation of the virus by disinfectants at room temperature

	A-11				**A-45**				**C-45**			
	1	10	30	60	1	10	30	60	1	10	30	60
Phenol	+	+	-	-	+	+	-	-	+	+	-	-
Formaline	-	-	-	-	-	-	-	-	-	-	-	-
Virkon(s)	+	+	+	-	+	+	+	-	+	+	+	-
Gluteraldahyde	+	+	-	-	+	+	-	-	+	+	-	-
Ethanol	+	+	+	-	+	+	+	-	+	+	+	-

Table 12: Concentration and purity of the isolates and positive control virus

Isolate	Concentration	Purity
A-1	29.4	1.6
A-11	63	1.4
A-45	89.1	0.81
C-1	43	0.93
C-45	67	1.6
Positive control	61	1.13

Fig. (1): Normal tissue culture of CEF

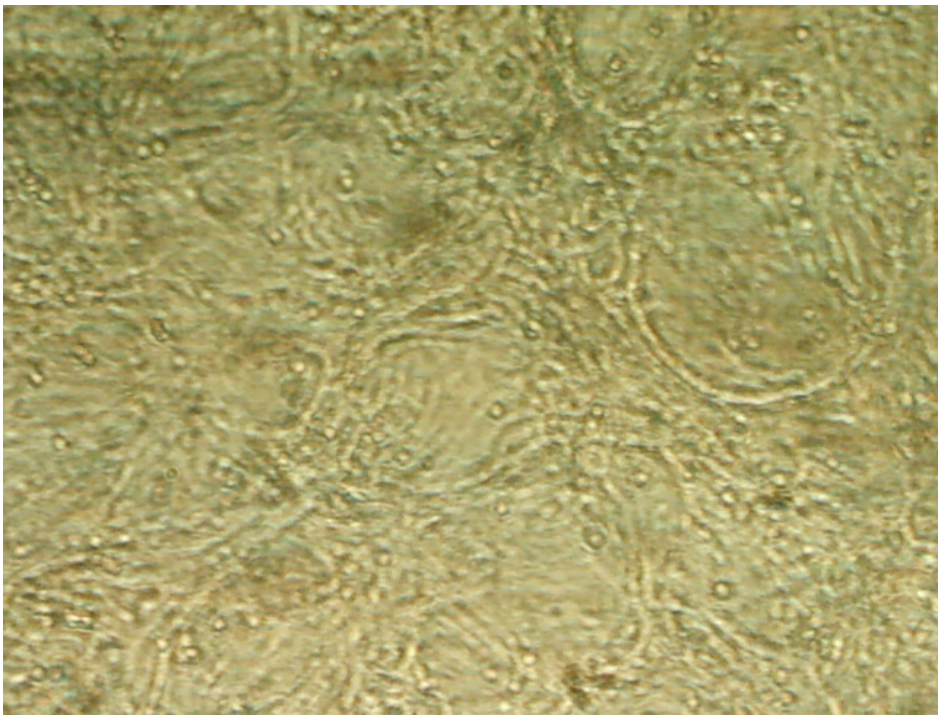


Fig. (2): CPE in CEF cells infected with the A-11 isolate

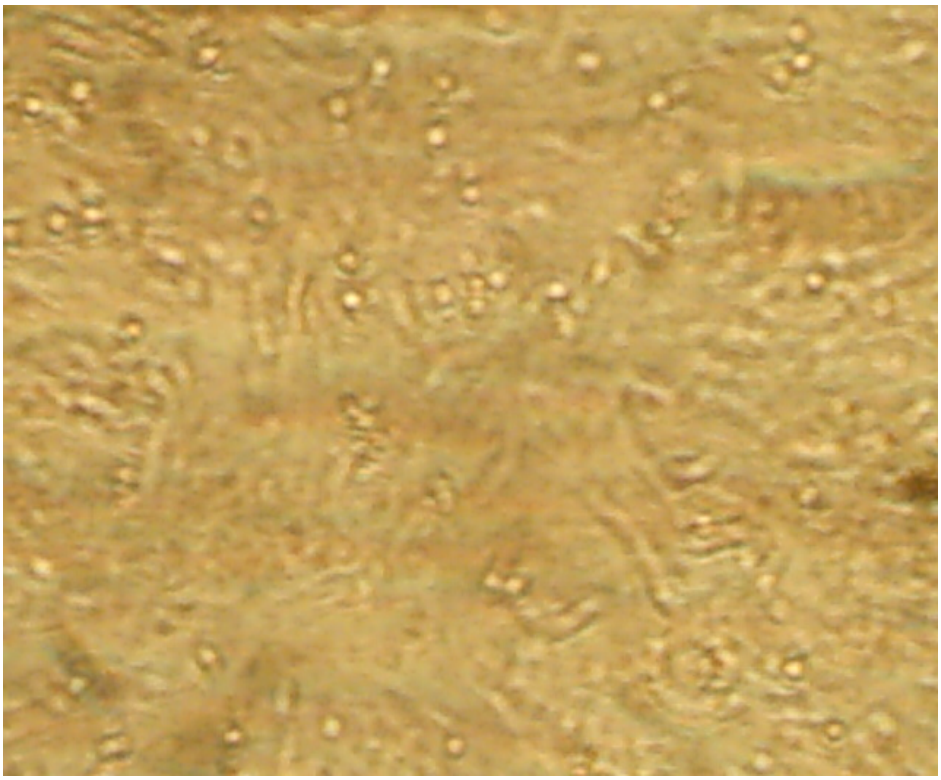
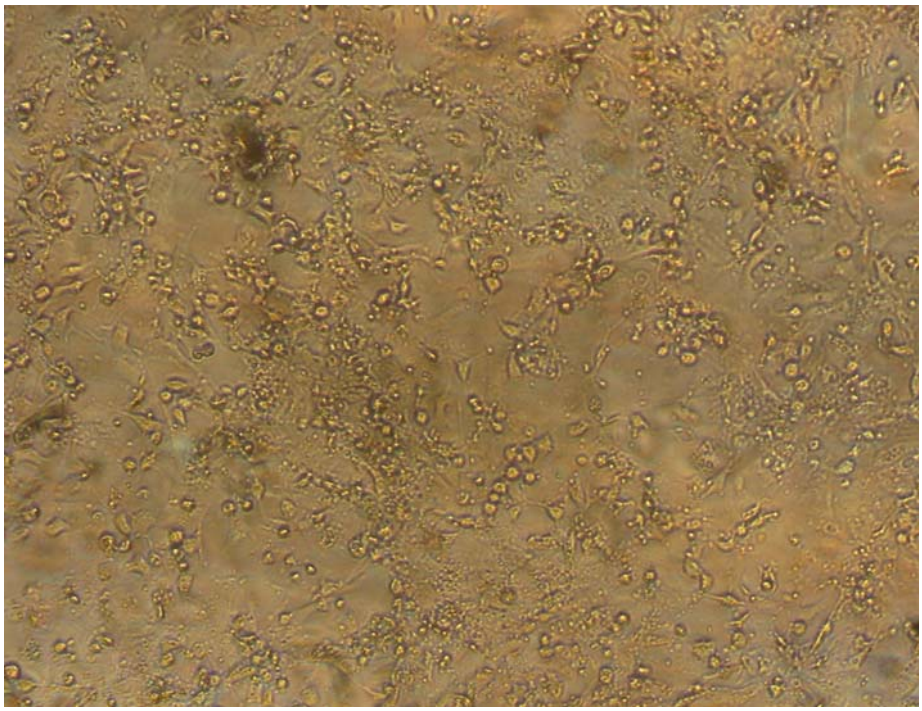


Fig. (3): Normal tissue culture of CKC

Fig. (4): CPE in CKC cells infected with the AI isolates



Fig(5): Plaque caused by A-45 isolate on CKC

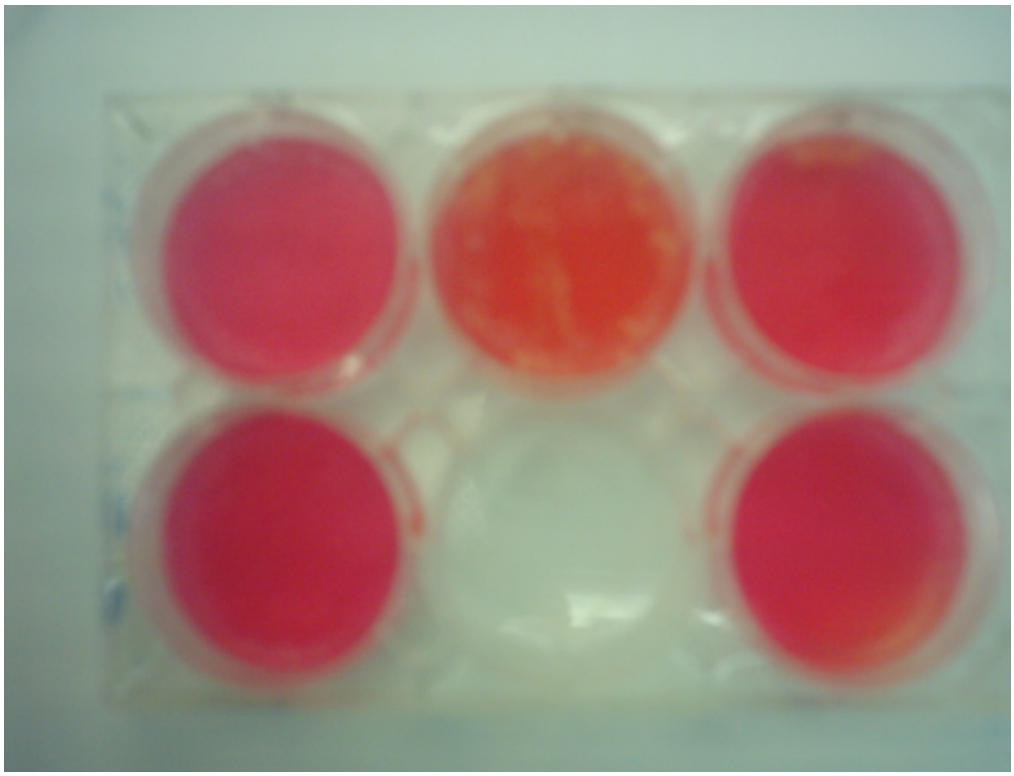
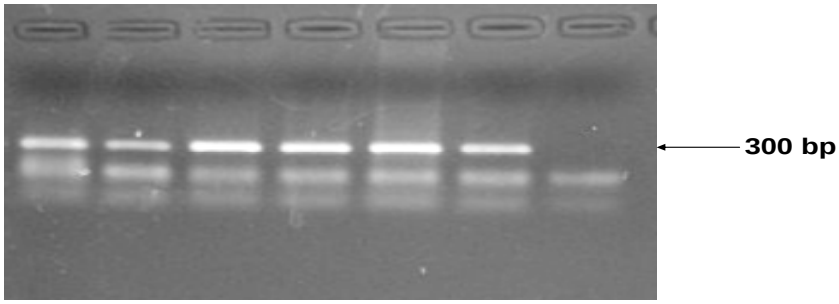


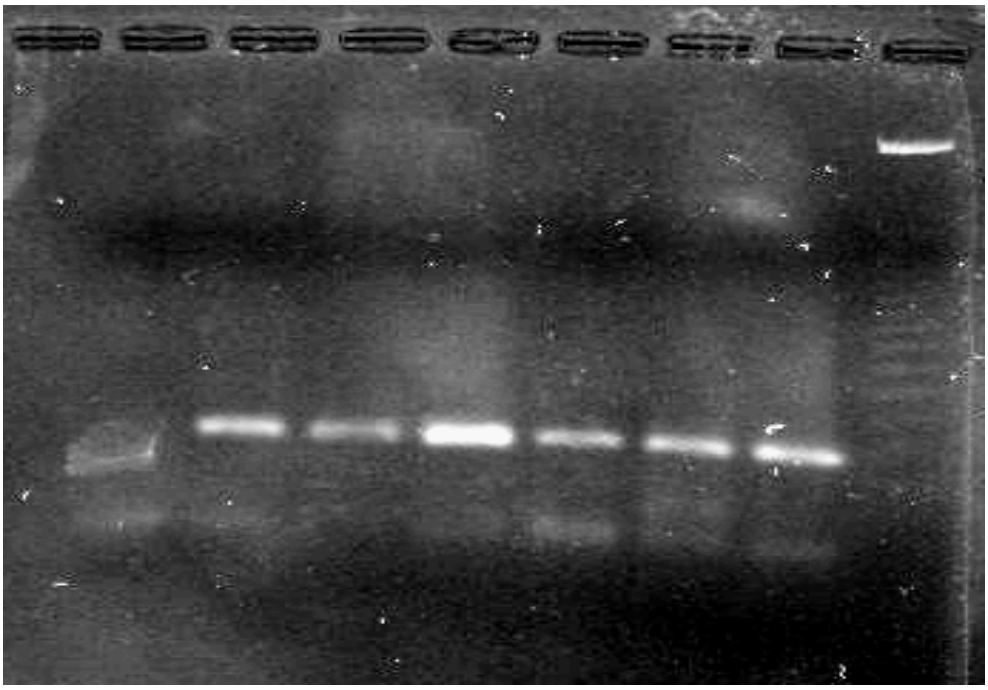
Fig. (6): Polymerase chain reaction amplification of isolated AI viruses



1 2 3 4 5 6 7

Lane 1- 5 field isolates, Lane 6 positive control and lane 7 negative control

Fig(7): PCR amplification pattern of isolates using H5 primers in 2 % agarose gel



1 2 3 4 5 6 7 8

Lane 1 Negative control

2 Positive control

3-7 field isolates

8 Molecular weight marker

CHAPTER FOUR

DISCUSSION

Avian influenza is one of the emerging diseases of zoonotic nature that threaten the livelihood of millions of small scale farmers. It is caused by influenza A viruses, which can affect a variety of domestic and wild bird species. Infection can range from asymptomatic to severe, depending on the virulence of the virus and the susceptibility of the avian host. It is defined as a notifiable disease by the OIE.A major concern was the possibility that the virus might spread to large populations. It became a disease of great importance for animal and human health. The study was carried out to determine the presence of antibodies to influenza virus, in addition to isolate, identify, characterize and determine pathogenicity of isolates.Also to the study of stability of the isolates to different temperatures and various disinfectants.

The current avian influenza outbreaks which started in Asia in 2004 are caused by a virus of the H5 subtype which was further characterized as of the N1 subtype that was responsible for some human deaths. There is a current worldwide focus on avian influenza and due to the implication of disease spread to humans, the disease is considered a priority. The rapid spread of AI between countries and into new species intensified the risk of a pandemic that may affect both humans and animals. This emphasizes the need of a global effort to provide early, rapid detection of the virus.

Few surveys have been carried out about the disease in poultry in Sudan to determine the prevalence of the disease. AI in the Sudan was reported for the first time in 1923 (Report of the Sudan Veterinary Service, 1923).However, serious attempts to study the disease in poultry were not made until 1985 when Alamin and Khier examined sera from large animals in Kassala region, and found that many animals from all species were positive to the AGID. Alamin (2000) was able to isolate the AI virus type A for the first time in the Sudan which probably belonged to the mild type as it was not fully characterized.

During this study an outbreak of AI occurred in 2006 in poultry farms in Khartoum State in winter, which agreed with Elamin (2000) who reported that outbreaks of the disease occurred in Khartoum in 1998 in winter.

During the present work three serological tests for antibody detection, ELISA, AGID and HI were used, isolation of AI virus was attempted from tissues cloacal and tracheal swabs.

A total of 250 samples consisting of tissues, cloacal and tracheal swabs were examined for the isolation of the virus. The virus isolation rate in the poultry farms after the outbreak from cloacal swabs were higher than those detected in tissue, and the rate of virus isolation was 20% from the tissue and 80% from cloacal swabs.

The isolates were identified as influenza virus type A by AGID and were sub typed using H5N3 and H7N1 antisera in the HI test. The five isolates were inhibited by AI antiserum to H5N3.

A total of 516 sera collected from different farms in Khartoum State before and after outbreak were examined by serological tests.

The results of tested sera using AGID showed that 34 were positive, 23(9%) before the outbreak and 11(4.2%) after the outbreak (Table3, 4).

The results of tested sera during the present work using the AGID showed lower antibody prevalence compared to those reported earlier by workers who used the same test. Although 9% and 4.2 % of the birds were found positive before and after the outbreak, Elamin (2000) reported that 18% of 100 birds were positive. The lower rate of positive result could be attributed to the fact that the samples were collected from apparently healthy poultry with no report of outbreaks. The positive rates dropped from 9% before the outbreak to 4.2 % after it and this might be due to the fact that all the exposed birds were killed after occurrence of the disease outbreaks.

A total of 516 sera were tested by ELISA among which 135 (26.2%) were positive. Elamin (2000) examined 120 sera from birds and found that 28.4% were positive. Wegdan et al., (2007) examined 1054 sera and found that 911(86.4%) were positive.

For subtyping the antibodies detected by ELISA and AGID using H5N3 and H7N1 antigens the results revealed that among sera collected during 2004-2005 17(60.7%) out of 94 were positive to H5N3 antigen and 11(39.3%) were positive to H7N1 antigen. Of a total of 41 serum samples collected from poultry in 2006 the results showed that only 6(14.6) were positive to H5N3 antigen and there is no antibody detected against H7N1.

The difficulties in control of AI in developed as, well as in developing countries is well documented. Biosecurity is regarded as the most important tool in controlling the disease. Economic losses to the poultry industry, food security in developing countries, the great threat to the human health and the possibility of the risk of the emergence of a new pandemic due to a new strain which may arise from the animal reservoir, because of all these the biological, biochemical and physiochemical characteristics of AI were studied. It is well known that the AIV is rather fragile in higher temperature; this was confirmed during this study as the virus was reported to be very sensitive to 70^0C for one minute.

Study on the stability of isolates to some various physical and chemical agents indicated that either 8% Formalin or 2%phenol were the most effective disinfectants for inactivation of influenza virus based on HA activity results. The use of Formalin would likely be less expensive, but has toxicity; phenol would be more suitable for sensitive environments and materials. Results also

indicated that the isolates were resistant to 1% Virkon$^{(s)}$, 2% Gluteraldahyde and 70% Ethanol for 5 min at room temperature.

Other physical properties were also investigated. The study indicated that the virus was very sensitive to 70^{0}C for 1 min, but it was resistant to 37 ^{0}C and 56 ^{0}C for 5 min, but they lost their effectivity at 30 min at 37^{0}C and 56^{0}C.

Attempts were made to develop a plaque test in Chick Embryo Fibroblast (CEF), Chick Kidney (CK) cell cultures. The data suggested that the chick embryo provides a very good isolation system. The isolates produced clear plaques of small size on primary CEF and CKC within 48 hr post inoculation without addition of trypsin. These results confirmed the virulence of the isolates according to OIE report that the virulent strains produce plaques in cell culture (OIE, 1996).

Methods effective for early, rapid detection and confirmation of diseases are recommended and performed according to the OIE guidelines.

Conventional microbiological assays should always be maintained to validate and guide further developments with the novel diagnostic techniques. In this contest, nucleic acid-based technologies have contributed considerably to the field of diagnosis. The PCR is considered as a major advance in the technology of nucleic acid detection, whereas real-time PCR (RRT-PCR) is a widely used technique which facilitates early, rapid detection of the viruses. It

involves the use of fluorescent probes to moniter the identity and amount of product at the end of each cycle and hence it is a quantitative PCR.

Conventional methods were used for virus isolation and were later confirmed by novel methods which involve the use of nucleic acid-base techniques, mainly RT-PCR. The virus is usually first isolated from pathological materials in embryonated chicken eggs, the methods takes about 4-7 days. This is followed by subtype identification using a battery of specific antibodies raised against the different hemagglutinin H (H1-H15) and neuraminidase N (N1-N9) proteins.

In spite of lack of adequate facilities for safety measures to handle the virus and the fact that these tests (HA, NA, HI, NI) have to be performed in specialized laboratories, but it was possible to perform the isolation steps successfully and with minimum but careful safety and biosecurity measures. The results obtained by the HA and HI tests which are considered as type – specific tests, are very good and five AI type A could be detected right after the first passage of the virus with relatively suitable HA and HI titers. No NA or NI was performed due to lack of materials. Mortality of inoculated chick-embryos took place 48 hours post inoculation.

The AGID was performed for group-specific identification of type A virus. It was a rapid, sensitive and specific test through which it was possible to detect 5 type A viruses among the field specimens tested.

For pathogenicity determination, the isolate is usually injected into 6-8 week-old susceptible chickens: according to OIE, mortality rate exceeding 75 % within 10 days indicated a highly pathogenic virus; this could not be performed due to lack of enough safety facilities and fear of causing hazards to both human and birds in the laboratory. Instead, the pathogenicity of the virus isolates was determined by propagation of the virus in cell culture and its plaquing in semi-solid media. All five isolates produce clear cytopathic effect (CPE) clear small plaques readily within 48 hours post inoculation. Plaque production by AIV indicates that the viruses are of the highly pathogenic type in nature. It was stated that highly pathogenic AIV strains produce plaques in cell culture while mild ones do not (OIE, 2003). Moreover, both CEF and CKC proved to be very good hosts for propagation of the AI virus.

The AI outbreak in Sudan in 2006 caused by type A avian influenza virus of H5N1 subtype demonstrated the need to reduce the time required for disease diagnosis and rapid detection of the virus. RT-PCR technique was used for characterization of the isolates and confirmation of the results that were obtained by conventional virus isolation (VI).Trizol method was used for extraction of the RNA from infected allantoic fluids (Ming et al., 2001) which was found to increase the sensitivity and quality of the RT-PCR assays, the results was similar to those obtained by Starick (2003). The results indicated that the five isolates were avian influenza type A subtype H5 when two

primers for H5 gene were used. The RT-PCR is found to take less time to characterize the virus compared to the conventional methods. The results of the RT-PCR compared with virus isolation and serological tests, confirmed that all isolates were found to be H5, therefore the sensitivity for the detection of AIV by RT-PCR and by conventional methods were found to be comparable, but the issue is rapid diagnosis achieved by using RT-PCR.

Conclusions and recommendations

The present work has confirmed the presence of AI disease in Khartoum State. It can be concluded that

1. The prevalence of AI disease was confirmed and that can be seen by isolation of AI from the outbreak that occurred in Khartoum State by serological surveillance during this study.
2. Extraction of AI antigen from 5 isolates in allantoic fluid by Trizol method is a reliable method.
3. The RT-PCR is a useful test for detection and confirmation of the the AI.
4. Chick Embryo Fibroblast (CEF) and Chick Kidney Cell (CKC) can be employed in isolation and plaque production of AIV.
5. AI virus was inactivated at all exposure times by 8 % formaline,and by heat at 70 ^{0}C for 5 min and 30 min at 56 ^{0}C.

Recommendations

1. Further serological surveillance to cover all States of Sudan has to be conducted to clarify the situation of the disease in the country.
2. Because of disease situation, AI vaccine is recommended to be introduced for control of the disease.
3. Rapid methods for diagnosis of AI are to be established and implemented, mainly nucleic acid base techniques.

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APPENDICES

Appendix I

Deionized distilled water:

Water was distilled to get rid of salts and passed through deionizer to get rid of ions. Deionized distilled water (DDW) was used in preparation of solutions besides washing of equipment.

Appendix II

HA, HI and tissue culture reagents and solutions

1. Normal saline

This was prepared by dissolving 0.85 gram of sodium chloride in 100 ml distilled water.

2. Phosphate Buffer Saline (PBS)

This was prepared as follow. (1 liter)

Solution A	Na CL	8.0 gm
	KCL	0.20 gm
	$Na_2HOP_4.2H_2O$	1.44 gm
	$KHPO_4$	0.2 gm
	Distilled water	800.0 ml
Solution B	Ca CL2	0.1 gm
	Distilled water	100.0 ml
Solution C	Mg $CL_2.6H_2O$	0.10 g
	Distilled water	100.0 ml

Solution A, B and C were autoclaved separately and left to cool. Then solution A was added to solution B then C and the mixture was made up to 1 liter with distilled water (Patrick *et al* , 2003).

3. Alsever's Solution

This was prepared as follow:

Dextrose	20.5 g
Na CL	4.2 g
Sodium Citrate	8.0 g
Citric acid	0.55 g

The mixture was completed to one liter with distilled water and autoclaved at 115 ºC for 10 minutes.

4. Phosphate diluent (PD)

NaCl	16.0 gm
K Cl	0.4 gm
Na2 HPO4	2.3 gm
KH2 PO4	0.4 gm

DDW complete to 2000 ml

The PD is used for the preparation of cell disperising solutions such as 0.25% trypsin.

Appendix III

Antibiotics

1. Stock Solution

1 gram streptomycin vial was dissolved in 5 ml distilled water. 10 I.U Penzyle Penicillin 1 vial was dissolved in 10 ml distilled water. 2 ml Gentamycin 1 vial was dissolved in 12 ml distilled water. Those were then distributed each in sterile vials in amounts of 2 ml in each vial and stored at -40ºC until used.

2. Working Solution

For suspension of material for chick inoculation.

Penicillin	10000 I.U/ ml	1 ml
Streptomycin		1 ml
Gentamycin		1 ml
Fungizon		1 ml
Complete to 50 ml by PBS		46 ml
Total		5o ml

Stored at - 40 ºC until used.

Appendix IV

Tissue culture media and additives

1. **Preparation of one litre of Growth Minimum Essential Medium (GMEM) X 5 Concentration (stock).**

0.5 gram of growth minimum essential medium was dissolved in one litre of DDW and sterilized by filtration. It was then dispensed in 0.5 litre amount and frozen at -20C^0.

2. Preparation of GMEM X1 Conc (one litre)

GMEM X5	200 ml
Lactalbumen hydrolysate 5%	025ml
Yeast extracts 1%	025ml
Antibiotics (penicillin, streptomycin, gentamycin, mycoststin)	004ml
$NaHCO_3$ 7.5%	008ml
DDW complete to	1000ml

3. Preparation of GMEM outgrowth Medium (100ml)

GMEM X1	85ml
Tryptose phosphate broth 5%	05ml
Calf serum	10ml
Total	100ml

4. Preparation of 2- fold GMEM (100ml)

GMEM X5 conc	40ml
Lactalbumin hydrolysate	05ml
Yeast extract	05ml
$NaHCO_3$ 7.5%	00.8ml
Antibiotics	00.6ml
DDw	48.6ml
Total	100ml
Bovine serum albumin	01ml

5. Preparation of first overlay medium (100ml)

I) 2- fold GMEM	90ml
TPB	05ml
Calf serum	05ml
II) 1.4 % agar in DDW	

Prepared by dissolving 1.4 grams of purified agar in 100 ml of DDW and boiling the mixture for 1 hour, then transferring it to a water bath at 43^0C.

Equal volume of I and II were mixed and used as a first overlay medium.

6. **Preparation of a second overlay medium**

This contained first overlay medium, 0.2 % bovine serum albumen (1% of a 2.5% stock solution).

7. Tissue culture additives

7.1 Preparation of Tryptose Phosphate Broth (TPB) 5% solution

(One litre)

Tryptose phosphate powder	29.5g
DDW	1000ml

Sterilized by autoclaving for 30 minutes at 15 Ib pressure.

7.2 Preparation of 7.5% $NaHCO_3$ solution (100ml)

$NaHCO_3$ powder	7.5g
DDW	100ml

Sterilized by autoclaving for 30 minutes at 15 Ib pressure .

7.3 Preparation of 5% lactalbumen hydrolysate solution (500ml)

Lactalbumen hydrolysate powder	25g
DDW	500ml

Sterilized by autoclaving

7.4 Preparation of 1% Yeast extract solution (500ml)

Yeast extract powder	5g
DDW	500ml

8. Cell-Dispersing solutions.

8.1 Preparation of 2.5% Trypsin (0.5 litre)

Trypsin powder	37.5 gm
PD solution	500.0 ml

The solution was filtered through a filter paper and finally sterilized by filteration.

8.2 Preparation of 0.25 trypsin solution

2.5% trypsin solution	10.0 ml
PD solution	90.0 ml

Appendix V

PCR reagent

1. Ethidium bromide

Ethidium bromide is a fluorescent dye able to detect DNA in agar gel electrophoresis. It was prepared in a concentration of 0.5 µg/ml in TBE buffer and stored in a confined light tight container brown bottle at room temperature. The ethidium bromide powder is powerful mutagen and toxic dye that gloves ought to be wear during working and dye or powder ought to be decontaminated after use.

2. PCR marker (DNA ladder):

50 bp consisted to 16 DNA fragments with size of 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650,700,750, and 800 bp. The marker was stored at -20^{o}C till used.

3. Preparation of 10X Tris Borate EDETA (TBE) one litre

Tris Base	108 g
Boric acid	55 g
EDETA	9.3 g
Distilled water to	1000 ml

The solution was sterilized by autoclaving and stored at room temperature. The buffer was diluted then and used as 1x.

4. Working solution (1x)

TBE stock solution	5 ml
Sterile distilled water	95 ml

Mixed well by agitation and the buffer was kept at room temperature until used as running buffer during electrophoresis run and as a solvent for agar gel electrophoresis powder.